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Anguillulina dipsaci from "tulip root" oats injuring seedlings of a seeds mixture.

By T. GOODEY, D.Sc.

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Amongst biological strains or races of the stem eelworm, *Anguillulina dipsaci*, some are comparatively polyphagous and are capable of causing disease in a number of different host plants whilst others are restricted to one or two hosts. The strain causing "tulip root" in oats is usually regarded as a restricted one being confined to oats or, as in Yorkshire, to oats and beans, in which infection takes place in the seedling stage and persists during the greater part of the life of the plant. Whether the parasite will attack other agricultural plants is a matter of some practical importance and since in a crop rotation a seeds mixture for ley production may follow oats, it was decided to test whether the parasite would infect plants comprising a typical seeds mixture. It was also hoped to determine whether any of the grasses or clovers would be more or less permanently affected and thus serve as reservoir hosts for the parasite and so enable it to tide over the period between successive oat crops. The present paper deals with pot experiments set up to test this matter as a result of which it has been found that the oat strain of the parasite is capable of causing serious injury to the young seedlings of a number of grasses and clovers of a seeds mixture.

Experimental.

The source of the infective inoculum consisted of diseased oat plants which had been slowly air dried for four months prior to use. The plants, of a variety "Black Supreme" showed evidence, when received, of much "tulip root" with swollen stem bases and twisted tillers, etc., and *A. dipsaci* was very plentiful in the affected tissues. Previous experience had shown the necessity for slowly drying such diseased plants in order to produce an infective inoculum from the material. Before use the dried plants were cut up into small pieces. The soil used was a loam which had been steam sterilized about 5 weeks previously. To 2 bushels of soil were added 3 oz. of superphosphate, 2 oz. of agricultural lime and sufficient silver sand to render it fairly open in texture. Ordinary 6 inch flower pots were used. Each pot to be inoculated with oat material was first filled with the sterilized soil to within about 2 inches of the top and the surface was levelled. On this was placed several pieces of the dried oat material; care being taken to use pieces of the swollen leaf and stem bases. A further one inch or so of soil was then placed on this and made firm and on this surface the seeds were sown. At the time of sowing a further small quantity of the inoculum, consisting of rubbed up fragments of diseased leaf bases, was added amongst the seeds and the whole was then covered with a shallow layer of sifted soil. For each species sown a control, uninoculated, pot was also set up. Two pots of oats, one inoculated and one uninoculated, were also included; the seeds being sown rather deeper than in the case of the smaller seeds.

Seeds of the following grasses and other ingredients of a seeds mixture were sown on 12 September 1940:—*Lolium perenne* L., (perennial rye-grass), *L. perenne* L., (perennial rye-grass, leafy strain), *Lolium italicum* A. Br., (Italian rye-grass), *Dactylis glomerata* L., (cocksfoot), *Phleum pratense* L., (timothy), *Poa trivialis* L., (rough-stalked meadow-grass), *Festuca pratensis* Huds., (meadow fescue), *Cynosurus cristatus* L., (crested dogstail), *Trifolium pratense* L., (English broad red clover), *T. pratense* L., (single cut red clover), *T. repens* L., (Dutch white clover), *Brassica Napus* L., (Dutch rape).

After sowing, all the pots were watered from below so as not to disturb the surface layers by overhead watering and were then placed in a bed of peat so as to conserve moisture and keep the roots cool.

Results.

The seeds in all cases germinated well both in the control and the inoculated pots. In the case of all the plants tested the seedlings growing in the inoculated soil showed more or less evidence of invasion by the eelworm whilst those growing in the controls were perfectly healthy and normal in appearance. The results of the examination of the seedlings of the various plants growing in the inoculated pots are set out below. Disease symptoms were apparent in these pots from a fortnight to three weeks from the date of sowing.

Avena sativa L., (oats).—All the seedlings were so seriously diseased that, in each case, the central shoot grew only about 1 to 1½ inches above soil level and had a brownish shrivelled tip. After a few more weeks all the plants were dead, thus proving that the inoculum used was highly infective to the original host plant.

Lolium italicum A. Br., (Italian rye-grass).—Many of the seedlings showed a yellow or light brownish discoloration of the first leaf which in most cases had a slightly twisted or curved appearance instead of being straight. Dissection of such seedlings revealed numerous adults and pre-adult larvae of *A. dipsaci* inside the coleoptile and within the tissues of the first leaf mainly towards its base. In the course of the next few weeks a good number of the affected seedlings were so weakened that they died out and the stand of plants in the inoculated pot was not nearly so good as in the control.

Lolium perenne L., (perennial rye-grass).—Some of the seedlings showed a brownish discoloration and distortion of the central leaf. One such plant on dissection had 5–6 adult *A. dipsaci* within the tissues of the leaf base. The weakening and thinning out of the seedlings was not so marked as in the case of *Lolium italicum* but the stand of plants was not so good as in the control pot.

L. perenne., (perennial rye-grass, leafy strain).—Some of the seedlings had an irregular appearance with the central leaf discoloured and twisted. One seedling was found, on dissection, to have 20–30 *A. dipsaci*, as well as numerous larvae in the tissues of the leaf base. The stand of plants was not much reduced as compared with the control.

Dactylis glomerata L., (cocksfoot).—Several of the seedlings showed distortion and discoloration of the central leaf. In one dissected plant there were found more than 20 adult and late larvae of *A. dipsaci* invading the tissues of the leaf base. During the autumn and winter there was some loss of the weakened plants so that the stand of plants was not so good as in the control pot.

Festuca pratensis Huds., (meadow fescue).—Some of the seedlings showed rather shortened and discoloured central leaves. Of two such plants dissected, one had 4 and the other 2 adult *A. dipsaci* within the tissues at the leaf base. During the autumn and winter there was a considerable thinning of the apparently weakened plants resulting in a poor stand as compared with the control pot. The plants remaining, however, were rather taller and sturdier in appearance than the control plants. This may have been due to their having more room in which to grow.

Phleum pratense L., (timothy).—The seedlings showed no obvious signs of discoloration and distortion of the central leaf. Three plants were lifted and dissected. One had 10–12 *A. dipsaci* inside the coleoptile and within the tissues of the leaf base; the second had 2–3 *A. dipsaci* and the third had none. There was some thinning of the plants during the autumn and winter as compared with the control.

Poa trivialis L., (rough-stalked meadow-grass).—Some of the seedlings exhibited discoloration and stunting of the central leaf. Two plants dissected each had 6–8 *A. dipsaci* within the tissues of the leaf base. The plants in the inoculated pot suffered practically no thinning during the autumn and winter and showed as good a stand as those of the control pot.

Cynosurus cristatus L., (crested dogstail).—A few of the seedlings showed a little distortion of the central leaf. One such plant when dissected had a rather inflated coleoptile within the tissues of which there were 10–12 *A. dipsaci*. Two other plants, however, when dissected failed to reveal any examples of the parasite. Very little thinning out of the plants occurred during the autumn and winter and the stand was practically as good as in the control.

Trifolium pratense L., (red clover).—Many of the seedlings had an unusual appearance with one cotyledon, or both, showing irregular discoloured markings on the surface and edges. There were in many cases lesions at the base of the short stalk of the cotyledons. One such seedling on dissection was found to have more than 20 *A. dipsaci*, adults and late larvae, in the tissues of one of the cotyledons with brownish lesions. The other cotyledon was normal in appearance and contained no parasites. In a lesion at the base of the stalk of the affected cotyledon there were 18–20 examples of *A. dipsaci*. During the course of the next few weeks it become noticeable that most of the seedlings in the inoculated pot were failing to develop the central leaves and in all cases the tissues of the growing point gradually withered and died. Early in the autumn all the seedlings in this pot died out. Seedlings in the control pot which developed to the second leaf stage also died out during the winter; probably not being old and strong enough to withstand frost but they survived much longer than those in the inoculated pot.

T. pratense L., (single cut red clover).—Many of the seedlings showed the same symptoms as those of broad red clover with irregular discoloured lesions of the cotyledons and cotyledon stalks. One such plant on dissection had 30–40 *A. dipsaci* adults and late larvae in a lesion at the base of the cotyledon stalk around the growing point. The parasites were also numerous in the irregular lesions of the cotyledons. Most of the seedlings died out with withering of the central growing tissues and practically all of them failed to produce the first true leaf. They all succumbed earlier than the well formed plants in the control pot.

T. repens L., (Dutch white clover).—The same remarks apply to this as to the two red clovers. Cotyledons and cotyledon stalks carried irregular brownish lesions in which the parasites occurred in good numbers. There was withering and death of the central tissues and none of the plants survived until the winter whereas the stand of plants in the control pot was good with first and second true leaves and much later survival than in the inoculated pot.

Brassica Napus L., (Dutch rape).—Practically all the seedlings in the inoculated pot showed some irregularity in the shape of one or both cotyledons. One such seedling on dissection had small irregular

cotyledons and an inflated lesion on the stem. In the latter there were 20–30 *A. dipsaci* some of which were adults and the rest larvae. The parasite also occurred in smaller numbers in the tissues of the cotyledons. Later on in these plants, as in the clovers, the tissues of the growing point became withered and failed to develop true leaves or such leaves as did grow were misshapen. The plants gradually died out during the autumn and winter so that finally there were none left. This was in marked contrast to the control pot in which a thick stand of plants grew and mostly persisted through the winter.

Examination of the various species of grasses persisting in the inoculated pots for the presence of any disease symptoms, such as stunting or distortion and swelling of leaf bases, was made during the early months of 1941. Except in one case, however, no signs of disease were found and the plants had a healthy appearance. When single plants were lifted and carefully dissected in water no examples of the parasite were found in them. Either such plants had originally remained free from infection or if infected had been able to tolerate and finally to outgrow or throw off any infection they may have had. The one exception was a plant of *Lolium perenne* (perennial rye-grass, leafy strain) which early in April, 1941, was found to be rather small and stunted. When dissected, from 20 to 30 adults and pre-adult larvae of *A. dipsaci* were found in the swollen bases of the central leaves.

Discussion.

From the general failure of the parasite to establish itself and persist beyond the early seedlings stage it may be concluded that none of the grasses of the seeds mixture is likely to constitute a reservoir host for the oat strain of the parasite. The principal damage caused by the latter is to the young seedling plants in the first 3 or 4 weeks of their growth and this calls for some further consideration in its practical aspects.

The infection resulting to a greater or less extent in all the grasses, clovers and rape seedlings was quite unexpected. In each case the inoculum of dried oat material evidently proved to be highly infective and, furthermore, it was in sufficiently close contact with the young plants to afford the parasites an opportunity of effecting an easy entry into them.

In the case of the clovers and rape none of the affected seedlings succeeded in producing their true leaves and all were finally killed. In the case of the grasses infection resulted in misshapen and twisted, discoloured central leaves followed, in many cases, by thinning out of the plants.

One may quite reasonably ask whether such injurious effects might occur under ordinary farm conditions. A seeds mixture for the production of a ley may very well follow oats in the course of a crop rotation. It may be sown amongst the oats which then serve as a nurse crop or later in the year when the oats have been harvested. In the former case should the oats become affected with " tulip root " it is quite possible that the seedlings of the seeds mixture might also suffer injury from eelworm attack though in this case it is natural to assume that the parasites would be likely to prefer oats to any unusual host plants.

It is, however, the later sowing of the seeds following a crop of oats affected with " tulip root " which calls for special consideration. For the young seedlings to become affected in the field it would seem necessary for the parasite to be present in the surface layers of the soil in which the seeds are sown. This would depend in large measure on the tillage or cultivation operations employed on the land after the oat crop had been taken. If the oat stubble was not ploughed under there is every likelihood of the eelworms remaining in the surface layers of the soil. Ploughing might, in fact, be purposely omitted so as not to disturb the soil too deeply for the preparation of a good firm seed bed. In this event the ground would probably be harrowed and scuffled so as to remove weeds and produce as good a seed bed as possible. Under such conditions it seems probable that the parasites would be present in the surface layers of the soil since large numbers of them would be dispersed in the broken down stem and leaf bases of the diseased oats. In this way it is probable that they would readily be able to infect the young seedlings of the seeds mixture and produce in them similar effects to those observed in the foregoing pot experiments.

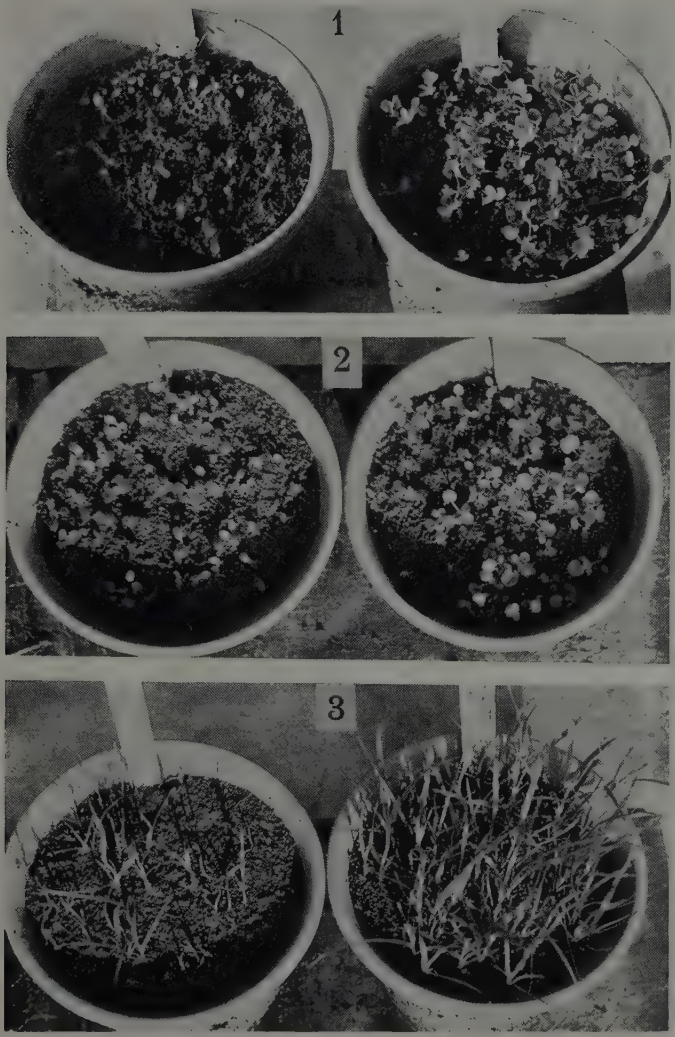
As a general recommendation, therefore, it may be suggested that where " tulip root " in oats has occurred the stubble should be ploughed under and buried as completely as possible so as to take the parasites from the surface layers of the soil and in this way avoid the risk of damage to the young seedlings of a seeds mixture.

The writer is indebted to Prof. R. T. Leiper for suggestions out of which the foregoing experiments arose, and to Dr. C. T. Calam for photographs.

Explanation of Plate.

Injurious effect of *Anguillulina dipsaci* from "tulip root" oats on seedlings of clover and grass photographed 1 month after sowing. Inoculated pots on left, uninoculated pots on right in each case.

1. *Trifolium pratense* L., single cut red clover.
2. *Trifolium repens* L., Dutch white clover.
3. *Lolium italicum* A. Br., Italian rye-grass.



Oat *Anguillulina dipsaci* in "Seeds" Seedlings.

[To face page 8].

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A Phenothiazine Experiment Statistically Treated.

By B. G. Peters, M.Sc., Ph.D., J. W. G. Leiper, M.R.C.V.S.
and
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(*From the Institute of Agricultural Parasitology, St. Albans.*)

Introduction.

In a previous paper, Peters & J. Leiper (1940) pleaded that, in anthelmintic experiments, the statistical problem of variation should be considered, not only so as to assess the significance of treatment differences after the results have come to hand, but also with a view to the prior construction of an experimental design capable of giving reliable answers to the questions posed. Reference was made to the "Enormous expenditure of time, labour and money" on anthelmintic experiments, the results of which usually remain undigested and of unknown significance.

While the paper was passing through the press a symposium on phenothiazine as an anthelmintic, led and summarized by Taylor & Sanderson (1940), was published in the Veterinary Record. The symposium deals with "Work carried out in various parts of the country and co-ordinated through the Agricultural Research Council"; it covers some 150 horses, 300 to 400 sheep, 70 to 80 goats, and a few cattle, pigs, and dogs. The drug was found to be remarkably efficient against horse strongyles and the stomach-worms of ruminants, and in varying degree less efficient against other worms.

Extensive tables of egg-counts, worm-counts, and weights of animals are published in the symposium. It was usual to count eggs or weigh animals once before treatment and several times afterwards. The repeated after-treatment determinations are made in order to detect a general trend in egg-counts or weights: in other words there is no attempt to measure and make allowance for the very considerable up-and-down variation from day to day. Peters & J. Leiper showed that this random variation affected a number of sheep together, so that the fact of using several animals does not smooth it out. This is confirmed

by the " Day Means " in Table VIII below, where the means for 18 lambs show remarkable fluctuations within each of the two treatment periods.

In some cases two or even three replicate counts are made, but in others one is left to assume that a single slide has been counted ; although the variation from slide to slide is known to be large, no attempt is made to measure it and allow for it. Similarly, the variation from one animal to another is one of the largest sources of variation in any experiment of this kind and it is for this reason that two or more animals are treated alike, yet in some of the experiments in this symposium the advantages of this replication are largely thrown away by the pernicious use of " composite " faecal samples, and in no case is this source of variation measured and allowed for.

It is small wonder then that, when the reader comes to consider the variation between treatments (between different rates of dosing, or between giving the dose singly or fractionally), there are no data with which to assess the significance of such differences. In an experiment of this kind the total variation of the experiment as a whole is made up of constituent variations such as day to day, animal to animal, slide to slide, and treatment to treatment ; only if the latter is significantly larger than any of the former can it be fair to consider the treatment differences as real. The research worker in anthelmintics has a plain duty to design his experiment in such a way that all relevant sources of variation can be measured ; he has a further duty to measure them and to allow for them in analysing his results. If he cannot or will not do this, he should get someone to do it for him.

It is not denied that some useful information has come from the symposium. But it is claimed that the information is vague, and that a much larger amount of precise information could have been had for the same expenditure of effort if only there had been genuine co-ordination and expert attention to experimental design. The resounding phrase : " Co-ordinated through the Agricultural Research Council " appears to mean little more than that a central supply of the drug was handed round to research institutes which then proceeded to exercise their own sturdy independence in the use of it.

One feature of the presentation of egg-counts in this and similar publications is liable to mislead the uninitiated : the actual counts are

multiplied by 100 to convert them into a basis of " Eggs per gramme." Thus, a single count of 6 before treatment, and 0 after treatment, would be printed as 600 and 0 respectively, which looks far more impressive than it really is. In fact, if successive counts were made by a perfect technique from a single sample of faecal emulsion having a mean of 300 eggs per gm., about 5 per cent. of such counts would be 0, and 5 per cent. would be 600 (*i.e.*, 6 actually counted) : only 22 per cent. of actual counts would be 3, the true value. This is merely the fundamental " error " variation from slide to slide : the up-and-down variations due to days and animals are far larger. We suggest that actual counts are preferable, especially where zero counts are included in a comparison. Provided all counts in one publication are on the same stated basis, there seems no special value attaching to " Eggs per gramme " ; alternatively, if the desire is to impress the reader, why not " Eggs per kilogramme " ?

Destructive criticism is, of course, insufficient. It happens that, long before the publication of the above symposium, we had in view the designing of an experiment to test phenothiazine as an anthelmintic in sheep. From the previous work of Peters & J. Leiper we had some idea of the sources of variation likely to be involved, and we were anxious to make due allowance for them in analysing our results. None of us is a statistician, but we have been most fortunate in having the advice of Prof. R. A. Fisher, F.R.S., regarding both the design and the handling of results ; we wish to tender him our thanks for assistance without which we should certainly have floundered.

It will be seen below that the experimental results were of little value except in a negative way ; but we were as much interested in establishing a satisfactory technique as in testing the drug, and it is from the former aspect that we feel our experiment worth describing.

Eggs and worms were counted by P. A. C., J. W. G. L., and G. C. Martin ; the general design (subsequently approved by Prof. Fisher) was due to J. W. G. L. and to B. G. P., who is responsible for statistical analysis, presentation, and the above critical remarks.

Technique.

The experiment, which lasted through July and August 1940, involved the use of a flock of 18 lambs born at Winches during the previous spring,

kept with their ewes, and exposed to equal chances of acquiring a natural infestation with helminths.

Dosage.—In view of previously published work, it was decided to test 0·3 gm. phenothiazine per Kg. body weight, making rather more than 10 gm. per lamb, and also half this dose. Taylor & Sanderson have since stated (1940) that " Ten grammes appears to be an adequate dose in sheep." The drug was to be given in a single dose in the form of a drench with water. There was also an untreated control group, making in all three groups for quantity of drug.

Methods of Administration.—The phenothiazine available was in the form of a fine green powder which was very resistant to wetting. With five times its weight of water and after prolonged and vigorous shaking it was possible to form a rather thick cream, which was used as a plain drench. Wetting the powder is facilitated by the use of substances which lower the surface tension, and of these we decided to use sodium glycocholate, and also hexylresorcinol. Following up a suggestion made to us verbally by W. P. Rogers, we considered it possible that the bile salt might have some anthelmintic effect of its own and it was therefore used at the relatively heavy rate of 0·3 gm./Kg. Hexylresorcinol is a known anthelmintic, and was used at the rate of 0·03 gm./Kg. Thus, there were three methods of administration : one a plain drench, one using bile salt as a wetter, and one using hexylresorcinol : the two latter methods gave much thinner emulsions with the same amount of water.

Criteria of Efficacy.—We decided to use egg-counts and weights of lambs, before and after treatment in both cases, and worm-counts *post mortem*, as criteria of the efficacy of treatment. Eggs were counted by the modified McMaster technique described by Peters & J. Leiper (1940, p. 128). Eggs from each lamb were counted on 3 different days before treatment and 3 days after, viz., the 5th, 12th and 19th days. On each day 9 counts were made for each lamb, 3 by each of 3 counters, making 972 counts in all. Lambs were weighed on a machine sensitive to $\frac{1}{4}$ lb. on or about the 6 days when faeces were collected for egg-counting. Worms were identified and counted in the usual way after *post mortem* examination, 3 weeks after treatment.

Experimental Design.—The three quantities of phenothiazine designated 0, 1, and 2, and the three methods of administration, designated A (plain drench), B (bile salt added), and C (hexylresorcinol added), take the

form of a 3×3 contingency table having 9 cells with 2 lambs in each cell, as shown in Table I. Thus, of the 6 control lambs given no phenothiazine, 2 received plain water, 2 bile salt solution, and 2 hexylresorcinol solution. The fundamental analysis of variance for the 18 lambs thus yields 2 degrees of freedom for "Quantities," 2 for "Methods," 4 for the interaction between them, and 9 for error, giving 17 in all.

Statistical Treatment.—Since the distribution of worms can be known only as it was *post mortem*, the only statistical treatment possible for worm-counts is the simple analysis of variance just referred to. For any given species of worm, if the variance for quantities or methods or both is significantly greater than the interaction variance, then it is permissible to compare the mean worm-counts per quantity (or method) and to use the error variance for assessing the significance of differences in the usual way.

TABLE I.
Experimental Design.

Quantity of Phenothiazine	0 (Control)	1 (0·15 gm./kg.)	2 (0·3 gm./kg.)	Method Totals.
Method of Administration	Number of Lambs Used			
A (Plain drench) ...	2	2	2	6
B (+Bile salt)	2	2	2	6
C (+Hexylresorcinol) ...	2	2	2	6
Quantity Totals	6	6	6	18

For egg-counts and weights of lambs, however, there are two symmetrical sets of data, respectively before and after treatment, and the problem is to make the best use of such data. We are indebted to Prof. Fisher for pointing out that the appropriate technique is the "Analysis of Covariance" described in Section 49.1 of his "Statistical Methods for Research Workers" (6th Edit., pp. 275–290). If X is any before-treatment value (or total or mean), and Y the corresponding after-treatment value, the ordinary analysis of the Y data will usually be far less sensitive than an analysis of the values $(Y-bX)$, where b is the regression coefficient of Y on X. The essence of the technique is the use of the "Error" sums of squares and products to calculate the regression of after-treatment on before-treatment data; for, putting the corresponding deviations as x and y, $b = \Sigma(xy)/\Sigma(x^2)$. The method is to analyse in three columns the

sums of squares for x and y and the sum of products xy, calculating b from the error component. It is unnecessary to calculate the individual data ($Y-bX$), or the individual deviations ($y-bx$) because, since :

$$(y-bx)^2=b^2x^2-2bxy+y^2,$$

the values in the x^2 column can be multiplied by b^2 and those in the xy column by $-2b$; horizontal summation over the three columns will then give the required analysis of the sum of squares of the adjusted values ($y-bx$). This transformation inevitably reduces the error squares, since a proportion of them is transferred to regression : squares due to other factors may be either increased or decreased.

Quite apart from the 36 sheep values, it is possible in the case of egg-counts to analyse the variation among the 972 individual counts, and this will provide information on the variation from day to day, between sheep, and between counters, besides testing agreement with the theoretical Poisson distribution.

Results.

(a) *Egg-counts per Sheep.*

War-time economy precludes publication of the 972 counts, which are filed at the Institute of Agricultural Parasitology. However, the total counts for each sheep, before and after treatment, are shown in Table II : since total counts are necessarily whole numbers whilst means often involve recurring decimals it is more accurate to work with the totals.

Table II.

Total Egg-counts per Sheep, Before and After Treatment.

Quantity	0		1		2		Method Totals
Method A ...	135	**271**	822	**277**	87	**64**	1,857
	105	**276**	187	**149**	521	**400**	**1,437**
Method B ...	90	**109**	331	**132**	112	**84**	1,634
	187	**359**	290	**348**	624	**236**	**1,268**
Method C ...	522	**771**	103	**112**	193	**242**	1,843
	426	**278**	331	**279**	268	**158**	**1,840**
Quantity Totals ...	1,465	**2,064**	2,064	**1,297**	1,805	**1,184**	5,334 **4,545**

(After-treatment totals are shown in bold type ; there are two lambs in each cell. The above totals should be multiplied by 7·41 to give "Eggs per gramme.")

The counts show that there is considerable variation from sheep to sheep, and that in some cases the count has increased after treatment. "Quantity" totals suggest that the controls have increased their egg output whereas the two treated groups have decreased theirs; oddly enough the decrease is more marked at the lower dosage rate. The nett effect over all sheep is a slight decrease after treatment, and this is reflected in the "Methods" totals where the decrease is greatest in the bile salt group.

Table III shows the analysis of squares of deviations before and after treatment (x^2 and y^2 respectively) and of product deviations (xy). From the error component is calculated the regression coefficient,

$$b=\frac{199,640}{466,271}=0\cdot4282.$$

TABLE III.

Analysis of Squares and Products, and of After-Treatment Variance.

Source	x^2	xy	y^2	D.F.	V.	R.
1. Quantities ...	30,082	— 40,520	76,414	2	38,207	1·966
2. Methods ...	5,200	+ 8,422	28,786	2	14,393	
3. Interaction ...	258,573	+112,767	77,731	4	19,433	
4. Error	466,271	+199,640	269,780	9	29,980	
5. Total	760,126	+280,309	452,711	17	26,630	

(D.F.=Degrees of Freedom; V.=Variance of y; R.=Variance Ratio. Standard Deviation=173·13 eggs per sheep. b=0·4282.)

In the same table the after-treatment squares (y^2) are divided by the available degrees of freedom to give the variance. From this analysis it is clear that methods of administration have a variance lower than those of interaction or error; differences between methods can therefore be ascribed wholly to chance. The quantity variance is higher than those of interaction or error, but not significantly so. Hence the after-treatment counts do not in themselves justify our assuming that phenothiazine has had any effect.

Taking now the before-treatment counts into consideration, by calculating the sum of squares $(y-bx)^2$ as described above, we get the analysis of variance of adjusted counts shown in Table IV, in which one degree of freedom for error has been used up in calculating b. In effect, the error variance has not been greatly reduced but the quantities variance

has been considerably increased. For the purpose of testing for significance, by using the variance ratio: Quantities/Error, it is necessary to allow for the random variation to which b itself is liable. This entails a reduced value for quantities, calculated as follows (see Table V). The

TABLE IV.

Analysis of Variance of Adjusted Counts, $Y-bX$.

Source	Sum of Squares	D.F.	Variance
1. Quantities ...	116,629	2	58,315
2. Methods ...	22,527	2	11,264
3. Interaction ...	28,554	4	7,139
4. Error	184,276	8	23,035
5. Total	351,986	16	21,999

Standard Deviation = 151·77 eggs per sheep.

values of x^2, xy, and y^2 in Table III for quantities and error are respectively summed. The reduced y^2 for error will be as in Table IV. The reduced y^2 for the sum is found by deducting from y^2 the value $\Sigma^2(xy)/\Sigma(x^2)$. The reduced y^2 for quantities is then found by difference. The resulting variances for quantities and error give a ratio of 2·407 which is not significant at the 5 per cent. point. Thus, although the analysis of covariance has given greatly improved values, they are not in this case sufficient to warrant the conclusion that phenothiazine has had any effect on egg-counts.

TABLE V.

Calculation of Reduced y^2 (Egg-counts).

	x^2	xy	y^2	Reduced y^2	D.F.	Variance	Ratio
Quantities	30,082	− 40,520	76,414	110,908	2	55,454	2·407
Error	466,271	+199,640	269,780	184,276	8	23,035	
	496,353	+159,120	346,194	295,184	10		

As stated above, the effect of calculating the regression coefficient is to reduce by unity the number of degrees of freedom for the component (namely, error) from which it was calculated. This usually results in transferring a significantly high proportion of the error variance to

regression. But in the present case, as Table VI shows, the regression variance is not significantly greater than the reduced error variance, so that no marked improvement could be expected.

TABLE VI.
Analysis of Residual Error.

	Sum of Squares.	D.F.	Variance.	Ratio
Regression	85,504	1	85,504	3·712
Error of Adjusted Counts ...	184,276	8	23,035	
Error of Unadjusted Counts	269,780	9		

(b) *Individual Egg-counts.*

The above analysis based on the 36 total egg-counts per sheep is adequate to testing for significance of treatment: in fact, if individual counts had been used instead, while the sums of squares and variances would of course have been different, the variance ratios relating to interaction would have been the same. But what has been called "Error Variance" above is actually the variance between the two sheep of each pair and, since it is based on totals covering three days and determined by three counters, it has concealed within it the variance due to sheep, days and counters. Thus, while the standard deviation of the above totals is 74 per cent. of the mean total, the standard deviation of individual counts is only 31 per cent. of the mean count.

Table VII gives an analysis of variance of individual counts so constructed as to give information on these other sources of variation. The principles governing the construction of such an analysis have been illustrated in Peters & J. Leiper 1940, pp. 129–132). The primary division of the data is into the two treatment periods, before and after treatment, after which the variation between days and between sheep, in each period separately, is required: interaction between days and sheep is used as a test of significance. The day and sheep variances are both significantly greater than their interaction variance, showing that these sources of variation have had a real effect.

Line 5 in the Table is the sum of the preceding four and is required merely as a factor for interaction with the variance for counters: so far as differences between the latter are concerned, variation due to sheep,

days, and treatment-periods is of no interest and they are all lumped together. The counter variance is, surprisingly, significant and there is a real but small difference (amounting to 0·846 of an egg) between the mean counts of J. W. G. L. and G. C. Martin. Unfortunately, the counting slide used by one of them was broken before it could be decided whether the difference was due to a small difference in volume between the two slides.

TABLE VII.
Analysis of Variance of 972 Counts.

Source	Sum of Squares	D.F.	Variance	Ratio
1. Treatment periods ...	640·45	1	640·4537	
2. Days per period ...	16,280·47	4	4,070·1175	$(\frac{3}{2})$ 8·058***
3. Sheep per period ...	44,919·80	34	1,321·1705	$(\frac{4}{3})$ 2·616**
4. Interaction (2×3) ...	34,348·38	68	505·1233	$(\frac{4}{8})$50·390***
5. Lots of 9 counts	96,189·10	107		
6. Counters	116·49	2	58·2448	$(\frac{8}{7})$ 3·308*
7. Interaction (5×6) ...	3,767·73	214	17·6062	$(\frac{7}{8})$ 1·756*
8. Error	6,495·67	648	10·0242	
9. Total	106,568·99	971	109·7520	

Standard Deviation=3·1661 eggs. Ratios starred are significant at the following points : *5% ; **1% ; *** 0·1%.

The error variance represents the variation between the counts for the three cells of a counting slide as determined by any one counter on any one day for any one sheep ; it amounts to 10·0242, giving a standard deviation of 3·1661 eggs per cell. The general mean for the whole experiment was 10·1636, and the close approximation to this of the error variance indicates very good agreement with the theoretical Poisson

TABLE VIII.
Mean Egg-counts for Days and Counters.

Day	Day Mean	Counter	Counter Mean
1.	6·3951	P.A.C.	10·2006
2.	7·8827	G.C.M.	9·7222
3.	18·6481	J.W.G.L.	10·5679
4.	6·9136	Critical Difference :	0·4975
5.	11·6111		
6.	9·5309		
Critical Difference :	0·7036	General Mean :	10·1636±0·1016

(Means for Sheep, Quantities, Methods, and Treatment periods are ascertainable from Table II.)

distribution and thus fully vindicates the adequacy of the counting technique. The total degrees of freedom are correctly one less than the 972 counts made.

Table VIII shows the various means for the experiment and also the appropriate critical differences, differences greater than which can be deemed significant.

(c) *Weight of Lambs.*

Each lamb was weighed on three different days before treatment and three after, not for evidence of a general trend in the weights within each period, but merely as a measure of the random up-and-down variation. Table IX, which is comparable with Table II, shows the mean weight

TABLE IX.

Mean Weights per Sheep (Kg.) Before and After Treatment.

Quantity	0		1		2		Method Means
Method A ...	47·00	**49·67**	41·47	**43·90**	34·23	**36·77**	39·23
	42·10	**42·43**	37·60	**40·97**	33·00	**34·03**	**41·29**
Method B ...	37·83	**38·83**	39·70	**43·00**	45·93	**48·73**	37·64
	35·63	**39·37**	33·30	**32·40**	33·43	**33·47**	**39·30**
Method C ...	41·87	**43·67**	37·37	**39·40**	36·60	**40·03**	37·91
	37·97	**42·40**	43·87	**38·43**	29·80	**32·37**	**39·38**
Quantity Means ...	40·40	**42·73**	38·88	**39·68**	35·50	**37·56**	38·261
							39·993

(After-treatment means are in bold type ; there are two lambs in each cell.)

for each lamb before and after treatment. These data have been treated in the same way as the total egg-counts, making use of the analysis of covariance. The analyses of variance for unadjusted and adjusted weights, given in Table X, show that this technique has resulted in a great reduction in all the variances. Analysis of residual error shows that regression has a variance over 28 times that ascribed to error in the adjusted weights, a ratio significant at the 0·1 per cent. point. Nevertheless, the quantities variance, from being 1·689 times as great as the interaction variance, has now been reduced to a value less than that of interaction, so that we cannot find that treatment has had any effect on the weight of lambs during the brief course of the experiment.

TABLE X.

A. Analysis of Variance of Unadjusted Weights, Y.

Source	Sum of Squares	D.F.	Variance
1. Quantities ...	80·7343	2	40·3672
2. Methods ...	15·2697	2	7·6349
3. Interaction ...	95·6029	4	23·9007
4. Error	235·8546	9	26·2061
5. Total	427·4615	17	25·1448

B. Analysis of Variance of Adjusted Weights, Y—bX.

1. Quantities ...	8·1611	2	4·0806
2. Methods ...	0·9184	2	0·4592
3. Interaction ...	27·7800	4	6·9450
4. Error	51·4280	8	6·4285
5. Total	88·2875	16	5·5180

Standard Deviations: Unadjusted, 5·1192 Kg.
Adjusted, 2·5354 Kg.

(d) *Worm-counts.*

The 18 lambs were killed 21 days after treatment and the gastrointestinal helminths were collected, identified, and counted. Large and infrequent species were counted directly; small and numerous trichostrongylids were estimated from aliquot parts. The counts cannot be detailed here, but are filed at the Institute of Agricultural Parasitology. Suffice it to give the mean count per lamb as follows. (i) Gastric and small-intestinal worms: Ostertagia 11,147; Cooperia 1,335; Trichostrongylus 780·5; Haemonchus 455·6; Nematodirus 290·3; Strongyloides 246·1; Capillaria 42·11; Moniezia 4·833; Bunostomum 1·556. (ii) Large-intestinal worms: Œsophagostomum 201·9; Chabertia 132·3; Trichuris 46·00.

Since worm-counts before treatment cannot be known, the covariance method is not applicable. Simple analyses of variance have been made for each of the above kinds, but in no case was the variance significantly great for quantity of phenothiazine or for method of administration. In fact, as often as not, the worm-counts for treated sheep were higher than those for the controls. Thus, the total of 264,300 worms of all kinds was distributed amongst methods of administration as follows: A (Plain drench) 84,441; B (Bile salt added) 89,927; C (Hexylresorcinol added)

89,932 ; and amongst quantities of phenothiazine as follows : 0 (Controls) 69,018 ; 1 (0·15 gm./Kg.) 99,821 ; 2 (0·3 gm./Kg.) 95,461. If one of the numerous helminthologists who have an unaccountable prejudice against statistical analysis should study these data he would doubtless conclude that plain water is a better anthelmintic than phenothiazine : actually the counts are well within the range of random variation. The above totals cover all worms, but the same general relationships between treatment groups apply also to Ostertagia and broadly to the other forms. It should perhaps be explained that although both adult and larval forms were counted, all the above data refer only to fully adult worms.

The standard deviation of worm-counts varies, according to the species of worm, from 80 to over 100 per cent. of the mean count. In view of this fact it is doubtful whether worm counts are worth while except where the treatment effect is very marked, or where large numbers of sheep are treated alike.

(e) *Correlation between Egg- and Worm-counts.*

We thought it interesting to see whether egg-counts and worm-counts revealed that high degree of correlation which they are commonly supposed to do. Egg-counts had been made on eggs of the thin-shelled type, but of course without differentiation into species. Worm-counts were on a species basis for males and a generic basis for females, except where only one species of a genus was present. By far the commonest genus of worms was Ostertagia, and these counts per sheep (divided by 1,000 to keep the data manageable) were correlated with the mean after-treatment egg-count per sheep, there being 18 pairs of values. The correlation coefficient was :

$$r = \cdot 4453 \pm 0 \cdot 2239 ;$$

$$t = \frac{r\sqrt{(n-2)}}{\sqrt{(1-r^2)}}, \quad = 1 \cdot 99,$$

which is not significant. There was therefore no correlation between numbers of Ostertagia *post mortem* and numbers of thin-shelled eggs found after treatment.

The above method leaves out of account the less frequent nematodes which also lay thin-shelled eggs, the difficulty of including them being that the " weighting coefficients " for eggs laid per worm per day are not accurately known. Kauzal (1933) has looked into this matter of weighting

coefficients and, partly following his guidance, the following coefficients were adopted : Nematodirus 1 ; Ostertagia, Trichostrongylus, Cooperia, and Strongyloides 10 ; Œsophagostomum, Chabertia, Haemonchus, and Bunostomum 100. The sum of the weighted worm-counts of all these genera (divided by 10,000) was then correlated with the mean after-treatment egg-count, giving :

$$r = \cdot 7430 \pm 0 \cdot 1674 ;$$
$$t = 4 \cdot 44,$$

which is highly significant.

There is here a statistical problem for solution : given the egg- and worm-counts, to find weighting coefficients for each worm such that the correlation coefficient is a maximum.

Discussion and Conclusions.

1. Powdered phenothiazine administered to lambs as a drench in doses of 0·3 gm./Kg. had no very marked effect on the worm burden as measured by egg-counts and no effect (in three weeks) on worm-counts and the weights of treated lambs. It may properly be objected that the experiment was on too small a scale to detect slight but real effects which may have been produced. This is theoretically true ; but effects which are too small to be revealed in 18 lambs by the sensitive statistical technique used are of no practical interest to sheep farmers.

2. Sodium glycocholate or hexylresorcinol greatly facilitates wetting the phenothiazine but had no effect either way upon any of the criteria.

3. Owing to the very high variability of worm-counts amongst lambs treated alike and previously exposed to equal chances of natural infestation, they are likely to be a waste of time except where large numbers of lambs are treated alike or where the treatment effect is very marked. An illustration of the latter is described by Taylor & Sanderson (1940, Table I, p. 640) where no stomach worms were found in 6 lambs given 15 gm. of Swales' phenothiazine mixture, but counts varying from 2,100 to 55,400 in 5 untreated controls.

4. In mixed infestations where eggs are not identifiable a significant correlation between worm- and egg-counts can be found if appropriate " weighting coefficients " are used for each kind of worm present.

5. Egg-counts and lamb-weights, determined on several occasions before and after treatment, readily lend themselves to statistical treatment by Fisher's " Analysis of Covariance " technique whereby the regression of after-treatment on before-treatment data is determined, and the variances relating to error and treatments are reduced to those relating to the corresponding deviations from the regression line. The usual effect of this method is to transfer a high proportion of the original error variance to regression, leaving only a small residual error ; this occurred with our weight data but not with our egg-counts.

6. Detailed analysis of individual egg-counts shows that the variances for days and for lambs are very high. It follows that adequate replication of these two factors is necessary. Probably not less than 4 lambs should be treated alike in any one treatment group, and eggs should be counted from each of these on at least 3 or 4 different days before and a similar number of times after treatment. On the other hand, now that the counting technique is known to give results in close conformity with Poisson theory, it is no longer necessary to make 9 counts from each lamb on each day : probably 3 would be sufficient.

7. There remains the difficulty of accounting for the discrepancy between our poor results and the good results claimed by some other workers using phenothiazine. The great majority of helminthologists decline to pay any serious attention to notions of variation or significance in their data, and in many cases no reliance can be placed on their extravagant claims owing to lack of effective controls, or to the use of insufficient replicates. In other cases there may be adequate data but they are neither published in detail nor statistically analysed ; they are therefore in effect barren. There is a small residue of quite startling cases where it is obvious without statistical analysis that phenothiazine has been highly satisfactory. It is in the face of these that our results are difficult to explain.

Possibly our sample of phenothiazine was at fault in some way. Fortunately there remains sufficient of it for comparison with other samples in future experiments. Possibly different flocks or breeds of sheep respond differently to treatment, or it may be that season of year or age of lambs is a controlling factor. Whatever the reason, we claim that it is important to realize that doses of over 10 gm. per lamb may be (as in our own case) almost without effect.

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POSTSCRIPT.

Prof. R. A. Fisher has seen the proofs of the above paper and has pointed out that, since the " Quantities " variance of Table V comes so near to significance and is in any case diluted by the presence of two rates of dosing, it would be fair to test the significance of the contrast between quantities 0 and 2 only. The effect of this is as follows (cf. Table V, p. 16) :—

	x^2	xy	y^2	Reduced y^2	D.F.	Variance	Ratio
Quantities	9,633	−24,933	64,533	85,901	1	85,901	3·7292
Error ...	466,271	199,640	269,780	184,276	8	23,035	
	475,904	174,707	334,313	270,177	9		

While the variance ratio is still not significant as such, since only one degree of freedom is involved the t-test is appropriate : $t = \sqrt{3{\cdot}7292}$, $= 1{\cdot}931$. And since only the positive tail of the distribution is required, one enters the table of t under probability $= {\cdot}1$, $n = 8$, where t is found to be 1·860. Hence we may conclude that the apparent positive anthelmintic effect would not occur by chance as often as once in 20 trials. We may now compare the adjusted " Quantity " totals, $Y - bX$, as follows :—

Quantity	0	1	2	Standard Error
Adjusted Total	1437	413	411	±372

(Since these are totals for 6 lambs, the standard error is the square root of 6 times the error variance.)

This leads to the conclusion that there has been a real reduction in the egg-counts due to phenothiazine and equally marked at the single as at the double dose. It remains true nevertheless that more worms were found post mortem in treated than in untreated lambs and that such a result is of no practical interest to sheep farmers.

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A Controlled Experiment with Phenothiazine in Sheep.

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Introduction.

In an experiment previously reported (1941) we were able to show little anthelmintic effect against the gastro-intestinal nematodes of sheep after using phenothiazine powder in single doses up to 0·3 gm. per Kg. body weight. In view of our experience and its contrast with that of many other workers, we felt it desirable to test a much larger dose, to compare it with another brand of the drug, and (because of the very large variation in worm burden from lamb to lamb) to use not less than four lambs in each treatment group.

Accordingly, 12 lambs from a flock known to be naturally infested were purchased last November, and these were divided at random into three groups as follows: A, untreated controls; B, given compressed 5 gm. tablets of phenothiazine (recently placed on the market by a well-known firm) at the rate of 5 gm. per 5 Kg. body weight; and C, given phenothiazine powder in water as a drench, at the same dosage rate, with the addition to each dose of 1 gm. sodium glycocholate as a wetting agent. This powder was from the same batch as that used in our previous experiment. In each case the full amount was given as a single dose.

The same three criteria of efficacy were used as before: three egg-countings and lamb-weighings before and after treatment, and post mortem worm-counts. Eggs were counted by the same modified McMaster technique, the individual counts representing the eggs in 0·005 gm. of faeces, but on this occasion only three cells were counted per sheep per day. We intended to kill only two of the four lambs in each

group, and to keep the remaining two under observation over a period of months. Unfortunately the " survivors " began to die off soon after the first half had been killed, the cause of death being apparently gastro-intestinal helminthiasis. Only worm-counts from lambs killed at the same time are comparable so that only 6 sets of worm-counts are available. The lambs were dosed on the 19/11/40 and half of them killed on the 17/12/40, 28 days later.

Statistically, egg-counts and lamb-weights have been subjected to analysis of covariance, as explained and discussed in our previous paper. The analysis of treatment-group totals is this time much simpler, the total 11 degrees of freedom being divisible into 2 for treatments and 9 for error.

Results.

The total egg-counts and mean lamb-weights for each lamb, before and after treatment, are combined in Table I. Below the data for the 4 lambs of each treatment-group are the total egg-counts and mean lamb-weights for the group as a whole, before and after treatment. Individual egg-counts and weights are filed at the Institute of Agricultural Parasitology.

Table I.

Total Egg-counts and Mean Lamb-weights for 12 Lambs Before and After Treatment (Weights in lbs.)

A. Controls.		B. Tablets Group.		C. Powder Group.	
Before	After	Before	After	Before	After
96 77·92	**84** 80·92	**100** 64·25	**49** 54·67	**121** 75·75	**17** 78·17
70 67·58	**27** 61·58	**146** 69·75	**40** 67·75	**128** 60·83	**12** 65·17
24 67·67	**23** 68·00	**114** 69·33	**41** 60·75	**138** 55·83	**20** 61·50
234 62·58	**202** 65·08	**55** 67·67	**22** 63·08	**271** 69·08	**59** 67·25
424 68·938	**336** 68·896	**415** 67·750	**152** 61·563	**658** 65·373	**108** 68·021

Total eggs before treatment : 1,497 ; After treatment : 596.
Mean weight before treatment : 67·353 ; After treatment : 66·160.
(Egg-counts are printed in Bold type.)

(a) *Total Egg-counts.*

The total egg-counts for each treatment-group show not only a marked fall after treatment in the two phenothiazine groups, but also a less marked fall in the untreated controls. A similar situation occurs in an experiment reported by Lapage (1940, p. 649) where falls in the egg-counts from two treated lambs were accompanied by a fall in that from the control ewe. Lapage draws attention to this point and states that " It must be considered," without explaining how. The fact is that analysis of covariance is fully adequate to dealing with any such general trend in the counts, upwards or downwards.

TABLE II.

Analyses of Unadjusted After-treatment Egg-counts, Reduced Counts and Residual Error.

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio
(*a*) Unadjusted After-treatment Egg-counts.				
1. Treatments	7,314·90	2	3,657·45	1·451
2. Error	22,681·57	9	2,520·17	
3. Total	29,996·47	11	2,726·95	
(*b*) Reduced Egg-counts.				
1. Treatments	15,230·69	2	7,615·35	11·273**
2. Error	5,404·46	8	675·56	
3. Total	20,635·15	10	2,063·52	
(*c*) Residual Error.				
1. Regression	17,277·11	1	17,277·11	25·575***
2. Adjusted Error ...	5,404·46	8	675·56	
3. Unadjusted Error ...	22,681·57	9		

Variance Ratio significant at 1% Point : ** ; At 0·1% Point : ***.
Standard Deviations : (*a*), 50·2013 eggs ; (*b*), 25·9915 eggs.

In Table II the analysis of variance of unadjusted after-treatment counts shows that the treatment variance is not significantly greater than the error variance, even at the 20% point. The after-treatment data alone, therefore, do not justify the conclusion that phenothiazine at 1 gm./Kg., tablet or powder, has had any effect on egg-counts.

The adjusted counts, obtained by eliminating from " Error " the effect of regression (of after-treatment on before-treatment counts) by analysis of covariance, are not published but are ascertainable from the data in Table I ; the regression coefficient was : $b=0{\cdot}6262$. As explained in our previous paper, the test for significance requires the use of a reduced variance for treatments, and this is given in Table II (b), which shows that the treatment variance is larger than the error variance with a high degree of significance. Thus, taking into consideration the before-treatment counts, we can conclude that such treatment results could have arisen by mere chance in less than 1 per cent. of similar trials and that the effect of phenothiazine must be judged as real. Statistically the reason for this improvement lies partly in the increased variance for treatments, but chiefly in the reduced variance for error : regression is responsible for 25 times as much of the original error variance as remains in residual error (Table II (c)).

TABLE III.
Egg-Counts : Comparison of Treatment Totals.

Treatment	Unadjusted Counts, Y.	Adjusted Counts, Y–bX	Differences	*Diff.* S.E.	Probability
A. (Controls)	336 eggs	+ 70·49	A–B : 178·36	2·426	·04
B. (Tablets)	152 ,,	−107·87	C–B : 196·17	2·668	·03
C. (Powder)	108 ,,	−304·04	A–C : 374·53	5·095	·001

We can now proceed to examine the significance of the treatment differences themselves, using the differences not between the actual counts, Y, but between the adjusted counts, Y–bX. Table III shows that all three of the possible comparisons are probably significant, and the difference between " Controls " and " Powder " is certainly so. Thus, the tablets have reduced the egg-counts in comparison with the controls, and the powder has reduced them in comparison with the tablets ; *i.e.*, powder is significantly more effective in reducing egg-counts than tablets. As compared with the controls, the results with powdered phenothiazine could have arisen by mere chance only about once in 1,000 similar trials.

We particularly wish to stress the fact that the after-treatment counts *alone* do not in this experiment reveal significant treatment differences ; these are brought out by the use of Fisher's " Analysis of Covariance " which validly takes into consideration the before-treatment counts also.

(b) *Individual Egg-counts.*

The total variance of the 216 counts made during the experiment (12 sheep with 3 counts each on each of 6 days) is analysed into its components in Table IV. The general mean count per 0·15 ml. of faecal suspension was 9·6898 eggs, being 13·8611 eggs before treatment and 5·5185 eggs after. The error variance, standing at 10·8657, is slightly higher than the mean count, but not sufficiently so to cast doubts on the adequacy of the technique of counting, which in any case was that used in our previous experiment where it was fully vindicated. The variances from sheep to sheep and from day to day, within each treatment-period, are significantly large as compared with their interaction, so that the totals or means for individual sheep or days could justifiably be compared if desired; *e.g.*, differences between any two sheep totals (within one treatment-period) in Table I greater than 32 are significant.

TABLE IV.
Analysis of Variance of Individual Egg-counts.

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio
1. Treatment Periods ...	3,758·3473	1	3,758·3473	
2. Sheep per Period ...	9,122·5448	22	414·6611	($\frac{2}{1}$) 38·16***
3. Days per Period ...	801·0775	4	200·2694	($\frac{3}{1}$) 4·02**
4. Interaction (2×3) ...	2,189·5909	44	49·7364	($\frac{4}{3}$) 4·58***
5. Error	1,564·6672	144	10·8657	
6. Total	17,436·2277	215	81·0987	

Variance Ratio significant at 1% Point: ** ; at 0·1% Point: ***.
Standard Deviation: 3·2963 eggs per 0·15 ml.

(c) *Weight of Lambs.*

The mean weight for each lamb, before and after treatment, is shown in Table I. Statistically, these data were treated in the same way as the total egg-counts were in Table II, and the results are shown in Table V. As in the case of egg-counts, the treatment variance for unadjusted after-treatment weights alone, is not significant; *i.e.*, the treatment means (Table I) of 68·896 lb., 61·563 lb. and 68·021 lb., respectively, reveal differences among themselves which, when compared with the variation between the four lambs of each group, must be considered to be random. When the before-treatment weights are brought into an analysis of

covariance, however, and the adjusted treatment variance is then reduced to allow for variation in the regression coefficient, the result is otherwise (Table V (b)). The treatment variance is now significantly greater than

TABLE V.

Analyses of Unadjusted After-treatment Lamb-weights, Reduced Weights and Residual Error.

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	Variance Ratio
(*a*) Unadjusted After-treatment Weights.				
1. Treatments	128·3038	2	64·1519	1·265
2. Error	456·5088	9	50·7232	
3. Total	584·8126	11	53·1648	
(*b*) Reduced Weights.				
1. Treatments	158·5077	2	79·2539	5·286*
2. Error	119·9413	8	14·9927	
3. Total	278·4490	10	27·8449	
(*c*) Residual Error.				
1. Regression	336·5675	1	336·5675	22·449**
2. Adjusted Error ...	119·9413	8	14·9927	
3. Unadjusted Error ...	456·5088	9		

Variance Ratio significant at 5% Point : * ; at 1% Point : **.
Standard Deviations : (*a*), 7·1220 lb. ; (*b*), 3·8720 lb.

the error variance, a significantly high proportion of the original error variance having been transferred to regression (Table V (c)). We are thus entitled to compare the adjusted treatment means (Y–*b*X) as shown in Table VI.

TABLE VI.

Weights : Comparison of Treatment Means.

Treatment	Unadjusted Weight, Y	Adjusted Weight, Y–*b*X	Differences	$\frac{Diff}{S.E.}$	Probability
A. (Controls)	68·896 lb.	+3·770	A–B : 6·210	2·268	·055
B. (Tablets)	61·563 ,,	−2·440	C–B : 8·703	3·179	·013
C. (Powder)	68·021 ,,	+6·263	C–A : 2·493	0·911	·390

The difference in mean weight between the powder group and the controls is of no significance : such a difference would arise by mere chance in 39 per cent. of similar trials. The controls appear to have gained more (or lost less) than the tablet group, but as such a difference would arise by chance in 5·5 per cent. of cases it is of doubtful significance. The powder group compared with the tablet group, however, gives a difference for which t has the low probability of 1·3 per cent. and we must judge this to be significant. Taking into account all three comparisons, it would appear that the use of powdered phenothiazine has had no effect on the weight of lambs during the brief course of the experiment, but that the use of tablets has caused a slight but significant set-back.

(d) *Worm-counts from 6 Lambs.*

As explained above, only 6 of the 12 lambs were slaughtered, one month after dosing, and the average count of fully adult worms per sheep was 24,027, made up as follows : Ostertagia, 6,833·3 ; Trichostrongylus, 16,050 ; Cooperia, 258·3 ; Strongyloides, 641·7 ; Nematodirus, 191·7 ; Haemonchus, 8·83 ; Bunostomum, 8·00 ; Chabertia, 23·67 ; Trichuris, 11·17. The mean worm-count per sheep (all worms) for each treatment-group was : A (Controls), 15,746 ; B (Tablets), 23,212 ; C (Powder), 33,123. Thus for the total counts the treated sheep carried more worms than the untreated, and this also applies to each of the frequent genera : Ostertagia, Trichostrongylus, Cooperia, and Strongyloides. The differences are, of course, ascribable to random variation, which is very considerable ; but it follows that worm-counts show no evidence of any anthelmintic effect from phenothiazine at 1 gm./Kg.

It is not appropriate to compare these worm-counts with the egg-counts discussed above, since the latter are based on all 12 lambs. If the egg-counts for the 6 slaughtered lambs alone are separately analysed, the differences between adjusted treatment totals are in the same sense as those based on the whole data, but are no longer significant. This is largely due to the fact that only 2 degrees of freedom are available for error. The total after-treatment egg-count for each treatment-group was : A (Controls), 107 ; B (Tablets), 62 ; and C (Powder), 76. For comparative purposes the controls may be reduced to 100, giving the following :

A (Controls), 100 ; B (Tablets), 58 ; C (Powder), 71.

Different species of worms have different rates of egg-laying, and these rates are not accurately known. But, using the weighting coefficients

suggested in our previous paper (which gave a significant correlation with egg-counts) and again reducing the controls to 100, the expected egg-counts (*i.e.*, on the basis of worm-counts) are :

A (Controls), 100 ; B (Tablets), 145 ; C (Powder), 208.

Thus, on the basis of the worms actually counted *post mortem* and using admittedly crude weighting coefficients, the tablets group gave only 40 per cent. of the expected egg-count, the powder group only 34 per cent.

This strongly suggests that phenothiazine has depressed egg-laying rather than removed the worms. The three after-treatment counts were made on the 13th, 15th and 17th days after dosing ; they were purposely left late so as to avoid any short-term effects of the drug on egg-laying, but to have left them later still would have been to risk including eggs from worms which had been picked up after dosing.

(e) *Powder versus Tablets.*

There can be little doubt that phenothiazine powder in the form of a drench is more effective in reducing egg-counts than the compressed tablets. This is not difficult to explain, for we have evidence that the tablets do not always break down readily. The lambs were dosed on the 19/11/40 and half were slaughtered on the 17/12/40. One of the survivors which had been given tablets died from helminthiasis on the 22/12/40, or 34 days after dosing, and in its abomasum were found three large fragments, parts of a single tablet, in all of which fragments the smooth outer surface of the original tablet was still clearly distinguishable. After being washed and dried these fragments weighed 4·5 gm., almost the weight of a whole tablet, or 15 per cent. of the dose originally given. Another tablet was placed in water to see how quickly it would break down into a fine powder ; at the end of 24 hours the experiment was discontinued, for although the tablet then showed a longitudinal crack there was no trace of real disintegration and no sign of solution. Yet these tablets are marketed by a reputable firm and recommended by them in the most glowing terms.

The effect of the tablets on lamb-weights, which were reduced in comparison with the powder-group by an amount which must be considered significant, is difficult to explain, especially as the tablets are expressly praised for their effect (over a longer period no doubt) in increasing weight-production. The probability of the difference having arisen by mere

chance is about 0·013, and of course it is always possible that ours was the one chance in 77. This is so unlikely, however, that it would probably be worth while to enquire further into this point. One thing is clear: there is no simple law relating the weight of lambs inversely to number of worms harboured, for the group with the largest weight-increase yielded the largest number of adult worms *post-mortem*.

Discussion and Conclusions.

1. As judged by worm-counts on six lambs, phenothiazine at the rate of 1 gm./Kg. body weight was without effect, whether given as compressed tablets or as a drench. Owing to the high variation in worm-counts from lambs treated alike, it is still possible that the drug had an anthelmintic effect which was too small to be detected: nevertheless, the untreated lambs contained fewer worms *post mortem* than the treated.

2. As judged by egg-counts per lamb on 12 lambs, before and after treatment, phenothiazine at the above rate significantly reduced the egg-output of the worms. Moreover, powdered phenothiazine given as a drench was significantly more effective than a commercial compressed product. Detailed comparison of egg-counts and worm-counts suggests that the effect of the drug at this rate is to reduce egg-laying *without* removing worms. If so, egg-counts can no longer be regarded as a safe index of efficacy unless the egg-counting is postponed until several weeks after dosing, when there is a danger that eggs from a new generation will complicate the picture. In the absence of accurate information on the laying rates of different worms, trials with lambs kept indoors and infested with pure lines suggest themselves.

3. As judged by lamb-weights before and after treatment, powdered phenothiazine at the above rate was without effect during the brief course of the experiment. The tablets, on the other hand, led to a significant set-back in weight, as compared with powder.

4. In comparison with a drench made up with powder, the 5 gm. tablets were much easier to administer, but they had a significantly inferior effect on egg-counts and lamb-weights, and at least in one case a tablet failed to disintegrate in five weeks.

5. Although our results are largely negative, from the aspect of statistical technique they form a good illustration of the value of "Analysis of Covariance" as applied to egg-counts and lamb-weights. In both

cases the after-treatment data could not disclose any significant differences between treatments : in both cases the inclusion of before-treatment data by this method resulted in the attainment of significance. We can at least hope that our work will be of some interest to other workers from this point of view.

6. Considering together all the criteria of efficacy and also the relatively high cost of the drug it is clear that, in the present experiment, a dose of 1 gm./Kg. gave results of no practical interest to sheep farmers. Since two brands of phenothiazine were used, it now seems unlikely that the discrepancy between others' results and ours can be ascribed to impurity of the drug. The conditions under which phenothiazine works well are obviously not yet understood, and for this reason it is to be hoped that negative or disappointing results will be fully reported by others. For the same reason we think it unfortunate that various research workers have seen fit to make premature and excessive claims on behalf of this new anthelmintic.

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Digestion in Parasitic Nematodes. II. The Digestion of Fats.

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In a recent paper Rogers (1940) discussed the digestion of carbohydrates by the parasitic Nematodes, *Ascaris* and *Strongylus*. In carrying this work further, the present investigation has entailed attempts to find quantitative differences in the ability of the two types of worms to digest foods as well as the qualitative study of the nature of the enzymes involved. *Ascaris lumbricoides* (pig strain) and *Strongylus edentatus* again provided contrasting forms of study. The former species is found free in the anterior section of the small intestine of the pig and lacking haemoglobin or its products in its intestinal contents (Liévre, 1934 and Rogers, 1940a) probably seldom feeds on host tissue. *Strongylus edentatus*, on the other hand, is found attached to the mucosa of the large intestine of the horse and ingests large quantities of the host blood and tissue (Rogers, 1940a, 1940b). Thus it is highly probable that *A. lumbricoides* feeds on simple digested material from the lumen of the host's intestine whereas *S. edentatus* derives nourishment from highly complex biological tissues. It was considered, therefore, that quantitative as well as qualitative study of the activity of the digestive enzymes of these two types of worms was necessary to throw light on the physiological relationship between the parasites and their hosts.

Literature dealing with Nematode digestive enzymes is scarce and has been briefly summarised in the previous paper of this series (Rogers, 1940). It should be noted, however, that Flury (1912) reported the presence of fat-splitting ferments in the tissue of *Ascaris*.

It must be emphasised that the experiments described in this paper were not designed to define precisely the nature of the lipases in *A. lumbricoides* and *S. edentatus*. Such an undertaking would be extremely difficult because the properties of lipases are greatly affected by associated substances i.e. the properties of a lipase from a given source may vary with the extent of purification. This applies to the optimum range of *p*H (Willstätter, Haurowitz and Memmen, 1924) and to the effect of bile salts (Willstätter and Memmen, 1924). Since only crude enzyme extracts were examined in these studies, care must be exercised in estimating the significance of the results obtained. However, this work was not undertaken purely as an investigation of parasite lipases. Rather, it was hoped that results might show roughly : (1) the relative activity of the lipolytic enzymes of the parasites concerned and thus indicate the importance of fats in their diets and (2) the effects of the ingestion of the hosts' tissue or intestinal contents on the activity of the lipases. Furthermore, as the enzyme preparations were crude extracts of intestines and their contents, it is probable that most of the substances normally present when the enzymes functioned in the living worms' intestines were also present when experiments were carried out *in vitro*. Indeed, if carefully purified lipases were used, the indications of what happened during digestion in the worms would have been even less accurate.

Method of Extracting the Enzymes.

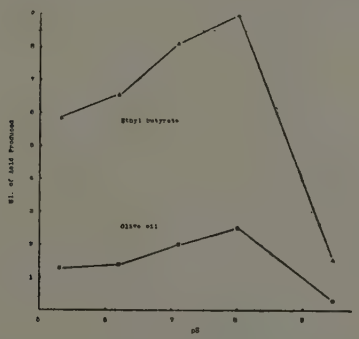
When qualitative results were required the procedure was the same as that detailed by Rogers (1940), the intestines of the parasites being dissected out, washed rapidly in distilled water and extracted with 50% glycerol in water.

To obtain quantitative results the worms to be used were washed several times with fresh saline, dried by rolling on filter paper and weighed. The intestines were then dissected from the worms as before, washed and ground up in a known volume of 50% glycerol. Using such enzyme extracts, it was possible to determine roughly the amounts of fatty acid produced per gram weight of worm during a known period of incubation.

Effects of Hydrogen Ion Concentration on Enzyme Activity.

The lipolytic actions of the parasite enzymes were examined at *p*H 5·3, 6·2, 7·1, 8·0 (Sørensen's phosphate buffers) and about 9·4 (bicarbonate-phosphate mixture). It was unfortunate that it was necessary to use $NaHCO_3$ solutions for this salt assisted the action of *Ascaris* enzymes

(Rogers, 1940) but borate buffers could not be used owing to the formation of the highly dissociated glyceroboric acid in the presence of glycerol. Small test tubes containing 0·5 ml. of buffer, 0·2 ml. of enzyme extract and 0·5 ml. of olive oil or ethyl butyrate were incubated at 37°C. for a known period, usually about 40 hours. (Before use, the ethyl butyrate was washed with Na_2CO_3 solution, separated, dried with anhydrous Na_2SO_4 and collected by fractional distillation between 119–121°C.) After incubation, the contents of the experimental tubes were titrated against N/100 NaOH (or, when bicarbonate was used, N/100 HCl) to the phenolphthalein end point. Control tubes were used to obtain the titre of the mixtures before hydrolysis, caused by enzyme action, had occurred.



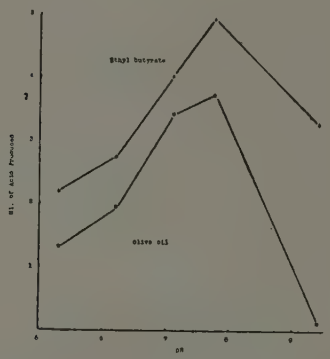
GRAPH I.—Showing the production of N/100 acid by *S. edentatus* enzymes acting on olive oil and ethyl butyrate at various hydrogen ion concentrations.

By subtracting the amounts necessary for the control titrations from the amounts used in titrating the incubated tubes, the volumes of N/100 acid formed by enzyme action were determined.

S. edentatus.—Tubes containing the enzyme extract, buffer and olive oil were incubated for 40 hours after which the titrations were carried out (before titration a strong unpleasant odour of fatty acids was noted). Similar tubes containing ethyl butyrate as the substrate for lipase action were also used. Graph I shows the results obtained, the curves giving the amounts of N/100 NaOH necessary to neutralise the acids produced at the different hydrogen ion concentrations. Several other lots of

S. edentatus which were used to test lipolytic activity all gave results similar to those shown in the curves. Invariably maximum activity was obtained at *p*H 8·0. Results at *p*H 9·4 varied somewhat (the bicarbonate-phosphate mixture was not always of the same concentration) but on no occasion was the activity greater than at *p*H 8·0. Indeed, enzyme activity at *p*H 9·4 was usually very slight.

A. lumbricoides.—Similar experiments using *Ascaris* enzyme extract showed maximum activity sometimes at *p*H 8·0 (see Graph II) and sometimes at *p*H 9·4. It was found that maximum activity was obtained in



GRAPH II.—Showing the production of N/100 acid by *A. lumbricoides* enzymes acting on olive oil and ethyl butyrate at various hydrogen ion concentrations.

the more alkaline solution when the bicarbonate was comparatively concentrated i.e. when the amount of N/100 HCl used in titrating the control tubes was more than about 5 ml. Since it was found (see later in this paper) that more concentrated solutions of bicarbonate vigorously activated the action of Ascaris enzyme, it was concluded that hydrogen ion concentration at about *p*H 9·4 did not assist the action of this enzyme as much as at *p*H 8·0 i.e. when more acid was produced at *p*H 9·4 it was considered to be due to the concentrated bicarbonate solution.

Effects of Bile Salts on Enzyme Activity.

Tubes containing 0·5 ml. of phosphate buffer (*p*H 8·0), 0·5 ml. of enzyme extract, 0·5 ml. of ethyl butyrate and 0·1 ml. of bile salt (both sodium glycocholate and sodium taurocholate were tested) of various concentrations were set up and incubated for about 30 hours at 37°C. The amounts of acid formed during this period were then determined by titration with N/100 NaOH. The results are summarised in Table I (*A. lumbricoides*) and Table II (*S. edentatus*).

Table I.

Showing the effects of bile salts of various concentrations on the activity of *A. lumbricoides* enzyme. The concentrations of the bile salts given are those of the final mixtures in the tubes.

Concentration of bile salt	Amount of N/100 NaOH used for titration	
	Sodium glycocholate	Sodium taurocholate
0·19%	1·21 ml.	1·33 ml.
0·16%	1·42 ml.	1·33 ml.
0·12%	1·39 ml.	1·30 ml.
0·09%	1·38 ml.	1·31 ml.

Table II.

Showing the effects of bile salts of various concentrations on the activity of *S. edentatus* enzyme. The concentrations of the bile salts given are those of the final mixtures in the tubes.

Concentration of bile salt	Amount of N/100 NaOH used for titration	
	Sodium glycocholate	Sodium taurocholate
0·19%	4·10 ml.	5·10 ml.
0·16%	4·45 ml.	5·22 ml.
0·12%	4·55 ml.	5·30 ml.
0·09%	4·90 ml.	5·50 ml.

Similar experiments were carried out using olive oil as a substrate for enzyme action. In proportion to the amounts of fatty acid formed, the effects of the bile salts were almost exactly the same though in the presence of sodium taurocholate of higher concentrations *A. lumbricoides* enzyme seemed a little more active.

Activation of *A. lumbricoides* Enzyme by $NaHCO_3$.

The production of acid from ethyl butyrate by *A. lumbricoides* enzyme extract in the presence of 3·75, 3·00, 2·25, 1·50 and 0·75% $NaHCO_3$ solutions and distilled water only, was examined after about 30 hours incubation at 37°C. Unincubated tubes were used for the control titrations. Results varied somewhat but, as a rule, enzyme activity was greatest in the presence of the higher concentrations of bicarbonate. Table III shows the results obtained from 3 sets of experiments.

Table III.

Showing the relative activity of *A. lumbricoides* enzyme extracts in relation to bicarbonate concentration.

$NaHCO_3$ concentration	Relative activity of *A. lumbricoides* lipase		
	I	II	III
3·75%	100	100	100
3·00%	92	37	98
2·25%	92	37	18
1·50%	68	79	16
0·75%	48	29	20
0%	4	5	3

Relative Rates of Olive Oil and Ethyl Butyrate Hydrolysis.

The relative amounts of acid formed by the action of *S. edentatus* and *A. lumbricoides* enzymes acting on olive oil and ethyl butyrate substrates were examined at *p*H 8·0. In each experiment, two tubes were used, both containing the same amount of enzyme extract taken from the one source and the same amount of buffer but one having olive oil as the substrate for enzyme action and the other ethyl butyrate. Five such experiments were carried out, each using enzyme extracted from a different lot of worms. The two tubes used in each experiment were then incubated at 37°C. for the same period (usually about 40 hours) after which titration with N/100 NaOH to the phenolphthalein end point was carried out. Control tubes were titrated as before and the amounts of acid formed during incubation obtained by calculation (see section on " Effects of Hydrogen Ion Concentration on Enzyme Activity ").

Table IV shows the proportions of the acids produced by enzyme action on olive oil and ethyl butyrate at *p*H 8·0, the amounts produced by the hydrolysis of olive oil being taken as unity.

The Relative Ability of *Ascaris* and *Strongylus* to Digest Fats.

Enzymes were extracted from the intestines of *A. lumbricoides* and *S. edentatus* by the quantitative method described previously. Tubes containing 0·2 ml. of enzyme extract, 0·5 ml. of buffer and 0·5 ml. of olive oil or ethyl butyrate were incubated for 44 hours at 37°C. after which acid production was estimated as before. Tubes buffered at *p*H 9·4 were not used as *A. lumbricoides* enzyme was unduly activated by $NaHCO_3$. Knowing the total weight of worms used to provide the enzyme under examination and the volume of 50% glycerol required for extraction, it was possible to calculate the amount of acid formed by enzyme action per gram of worm. In Tables V and VI the amounts of acid produced by the parasite enzymes per gram of worm, in 44 hours, are compared. The "relative activity" factors shown in the tables were found by dividing the amounts of acid produced per gram of *S. edentatus* by the amounts produced per gram of *A. lumbricoides* and they therefore show the relative ability of the parasites to digest fats.

Discussion.

Both esterase and lipase action have been demonstrated in the parasite enzyme extracts. It is difficult to decide whether these actions were due entirely to lipases or esterases or to combinations of both types of enzymes. Lipases would be capable of hydrolysing esters of polyhydric alcohols and higher fatty acids such as olive oil but could also hydrolyse the ester of a simple organic acid and a monohydric alcohol such as ethyl butyrate (Mann and Saunders, 1936, page 358). In this case, hydrolysis due to the latter action would be very slow. Reference to Table IV shows that acid production from ethyl butyrate was at least 1·33 times as great as the production of acid from olive oil. Hence it appears that lipases alone could not have been responsible for the results obtained. On the other hand, an esterase would only cause a slight hydrolysis of olive oil (Mann and Saunders, 1936, page 358) and it can be seen (Table IV) that the rate of hydrolysis of this substance was quite considerable.

TABLE IV.

Showing the relative amounts of acid produced from olive oil and ethyl butyrate by enzymes extracted from *A. lumbricoides* and *S. edentatus*.

Source of enzyme extract.	Amount of N/100 acid produced from olive oil.	Amount of N/100 acid produced from ethyl butyrate
A. lumbricoides	1 ml.	1·33 ml.
,, ...	1 ml.	1·56 ml.
,, ...	1 ml.	1·42 ml.
S. edentatus	1 ml.	3·98 ml.
,, ...	1 ml.	3·55 ml.

TABLE V.

Showing the amounts of acid produced from ethyl butyrate after 44 hours incubation at 37°C. by enzymes extracted from the intestines from 1 gram weights of worm tissue. For further explanation see text.

	*p*H	Amounts of N/100 acid produced from ethyl butyrate		Relative activity factors
		A. lumbricoides	*S. edentatus*	
No. 1.	5·3 ...	2·29 ml.	27·42 ml.	11·9
	6·2 ...	2·85 ml.	27·42 ml.	9·6
	7·1 ...	4·22 ml.	43·40 ml.	10·3
	8·0 ...	5·16 ml.	48·30 ml.	9·4
No. 2.	5·3 ...	2·37 ml.	46·01 ml.	19·4
	6·2 ...	2·43 ml.	56·10 ml.	23·1
	7·1 ...	4·44 ml.	78·80 ml.	17·7
	8·0 ...	5·71 ml.	97·91 ml.	17·1

TABLE VI.

Showing the amounts of acid produced from olive oil after 44 hours incubation at 37°C. by enzyme extracted from the intestines from 1 gram weights of worm tissue. For further explanation see text.

	*p*H	Amounts of N/100 acid produced from olive oil.		Relative activity factors.
		A. lumbricoides	*S. edentatus.*	
No. 1.	5·3 ...	1·41 ml.	2·31 ml.	1·6
	6·2 ...	2·03 ml.	2·60 ml.	1·3
	7·1 ...	3·61 ml.	12·14 ml.	3·4
	8·0 ...	3·93 ml.	12·14 ml.	3·1
No. 2.	5·3 ...	1·21 ml.	14·55 ml.	12·0
	6·2 ...	1·56 ml.	14·82 ml.	9·5
	7·1 ...	3·41 ml.	22·20 ml.	6·5
	8·0 ...	4·15 ml.	27·30 ml.	6·6

It appears, therefore, that an esterase alone would not give rise to the results shown. Furthermore, although only 5 sets of experiments were carried out, it seems reasonable to conclude that the relative rates of hydrolysis were fairly constant and, in relationship to its effect on ethyl butyrate, *A. lumbricoides* enzyme extract consistently digested about $2\frac{1}{2}$ times as much olive oil as *S. edentatus* enzyme (see Table IV).

These results lead to the conclusion that both esterases and lipases were present in the intestines of *A. lumbricoides* and *S. edentatus*. In both cases, esterase was the most active enzyme but the relative amounts of esterase and lipase varied in the two parasites, the relative proportion of esterase being greatest in *Strongylus*.

It may be thought that the relative production of acid from ethyl butyrate and olive oil was due to the experimental procedure adopted. Thus, as lipolytic action is usually reversible and glycerol (a product of the digestion of olive oil) was present in the enzyme extract, it may be considered that lipase action was brought to an equilibrium earlier than when ethyl butyrate was used as a substrate. This would give rise to a false low production of acid from olive oil, especially if the period of incubation was long. However, care was taken to ensure that a large excess of olive oil over glycerol was present (actually the ratio must have been greater than 5 : 1) in the experimental tubes and the time of incubation was such that only a small amount of oil was hydrolysed. It seems, therefore, that adequate precautions were taken to prevent equilibrium being reached before the experiments were terminated.

Evidently the lipolytic enzymes from *S. edentatus* and *A. lumbricoides* were similar in some aspects yet differed in others. As far as hydrogen ion concentration was concerned, all reached a maximum efficiency at *p*H 8·0. It may be thought that *S. edentatus* enzyme was activated by phosphate and that the fall in activity in the region of *p*H 9·4 was due to the lack of this salt. Actually, to avoid this error, some phosphate was added to the bicarbonate used in the most alkaline solutions. The doubt as to the relation of *A. lumbricoides* lipase and esterase to *p*H has largely been removed by the examination of enzyme extracts in the presence of $NaHCO_3$ solutions of various concentrations. It appears, therefore, that the similarity of the effects of *p*H on the parasites' enzymes was reasonably established.

In relation to the presence of $NaHCO_3$, the enzymes obviously differed, This salt tremendously activated *A. lumbricoides* enzymes, whereas it did

not appear to affect the action of *S. edentatus* enzymes except in so far as *p*H was concerned, when activity was reduced.

Bile salts showed different effects on the lipolytic enzymes. In the case of *S. edentatus*, both salts retarded fat digestion, the higher concentrations having a greater retarding effect than the lower concentrations (see Table II). *A. lumbricoides* enzymes were assisted by sodium glycocholate, the maximum effect being observed when the tubes contained a 0·16% solution of the salt (see Table I). Sodium taurocholate, on the other hand, retarded fat digestion in this parasite. Evidently the lipolytic enzymes from the two parasites differed in their relationship to sodium glycocholate.

Briefly, then, the enzymes of the two parasites were similar in their reactions to *p*H but differed in their reactions to $NaHCO_3$ and bile salts. No distinctions between the reactions of the esterase and lipase in each parasite could be detected. This fact lends some doubt to the supposition that both esterase and lipase were present in the worms.

S. edentatus undoubtedly showed far greater ability to digest fats, this being especially marked when esterase ability was examined. Table V shows that enzyme from 1 grm. of *Strongylus* was capable of hydrolysing from 9·4 to 23·1 times as much ethyl butyrate as enzyme from 1 grm. of *Ascaris*. Olive oil was also hydrolysed in greater quantities by *S. edentatus* but the "relative activity" factors were not so large in this case. These comparisons refer only to digestion carried out over a period of 44 hours but as it was found that the rate of digestion of olive oil was almost a linear function of time for about the first 50 hours of incubation, the "relative activity" factors are probably correct for all periods of time up to that time.

It cannot be definitely claimed that the enzymes extracted from the parasites were not simply absorbed from the hosts' ingesta. Certainly in the case of *S. edentatus*, this is highly improbable as the diet of this parasite seems to be entirely composed of host blood and tissue and the reactions of the enzymes to bile salts were not those of normal lipase. Again, the digestion by enzymes from the intestines of 1 grm. of *Ascaris* was so constant that it seems unlikely that the enzymes were obtained from a variable outside source. Furthermore, it would be expected that the quantity of material ingested by the worms would vary from time to time and this would cause variations in enzyme activity per grm. of worm if the enzymes were obtained from the hosts' ingesta.

Assuming that the enzymes examined were those of the parasites, it is evident that digestion in the worms was such that the ingestion of the host blood and tissue in the case of *Strongylus* and the intestinal contents in the case of *Ascaris* would provide suitable media for lipase and esterase action. Thus the parasites of the horse would ingest material of *p*H 7·2–7·5 (Brey, 1926) and probably little bile salt would be taken from the host's ingesta to retard enzyme activity. *Ascaris*, absorbing material from the small intestine of the pig, would obtain large quantities of bicarbonate secreted from the duodenal glands, and sodium glycocholate (sodium taurocholate is absent from the bile of pigs, Hammersten and Hedin, 1914) in fluids of a suitable *p*H (Long and Fenger, 1917). Such a diet would therefore provide an excellent medium for esterase and lipase action.

The results showing the relative ability of the two species of parasites to digest fats indicate that *A. lumbricoides* may absorb fats already digested by the hosts' enzymes. Of course digestion by *Ascaris* would be improved by the presence of bicarbonate and glycocholate but even then it appears that *Strongylus* would be more efficient. Indeed, the observation that *S. edentatus* is a tissue feeder and *A. lumbricoides* ingest partly digested material seems to be upheld by the examination of the ability of the two worms to digest fats.

Summary.

1. Enzymes hydrolysing olive oil and ethyl butyrate (probably lipases and esterases) have been extracted from the intestines of *Strongylus edentatus* and *Ascaris lumbricoides* (pig strain).

2. Maximum enzyme activity was obtained at *p*H 8·0.

3. *A. lumbricoides* enzymes were assisted by sodium glycocholate. Sodium taurocholate had a retarding effect on the enzymes from both parasites.

4. Enzymes extracted from *A. lumbricoides* were activated by the presence of $NaHCO_3$ solutions whereas *S. edentatus* enzymes were not activated by this salt.

5. Esterase action, relative to lipase action, was $2\frac{1}{2}$ times greater in *S. edentatus* than in *A. lumbricoides*.

6. Per gram of worm, esterase activity in *S. edentatus* was 9·4 to 23·1 times as great as in *A. lumbricoides*. The ability (per gram of worm) to hydrolyse olive oil was 1·3 to 12·0 times greater in *S. edentatus*.

7. It is concluded that the lipolytic enzymes in *S. edentatus* differ from those of its host and from those of *A. lumbricoides*. The nature of the parasites' enzymes is such that the ingestion of host blood and tissue (*S. edentatus*) and host ingesta from the small intestine (*A. lumbricoides*) would provide media in which the lipases and esterases would be highly active.

Acknowledgments.

The author is indebted to Professor R. T. Leiper for criticism and advice. Thanks are also due to Mrs. L. E. Rogers, M.Sc., who assisted in the preparation of the enzyme extracts.

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Digestion in Parasitic Nematodes. III. The Digestion of Proteins.

By W. P. Rogers, M.Sc., Ph.D.

(*From the Institute of Agricultural Parasitology, St. Albans.*)

Previous papers by Rogers (1940a and 1941) have dealt with the digestion of carbohydrates and fats by *Ascaris lumbricoides* and *Strongylus edentatus*. The nature of the proteolytic enzymes and the comparative activity of these enzymes in the same parasites is considered in this paper. Literature dealing with work of this type has been reviewed in the previous publication (Rogers, 1941) of this series.

The procedure followed in preparing enzyme extracts both for quantitative and qualitative work was the same as that described by Rogers (1941).

Nature of the Proteolytic Enzymes.

The investigation of peptic and tryptic digestion by the Henriques-Sørensen (1909) formol titration method has indicated a means of determining the nature of protein digestion by unknown enzymes (S. W. Cole, quoted by Wigglesworth, 1928). Tubes, one containing mammalian pepsin, albumin and HCl-KCl buffer (pH 1·4) and the other mammalian trypsin, albumin and phosphate buffer (pH 7·1) were incubated at 37°C. At regular intervals, 0·5 ml. of the mixtures were withdrawn and the " free " acid titrated to the phenolphthalein end point with N/100 NaOH, after which 5·0 ml. of " neutral formol solution " was added to each. (The " neutral formol solution " was a 40% formaldehyde solution to which phenolphthalein and enough NaOH had been added to make the solution faintly pink in colour.)

The methylation of the amino acids formed by the action of the proteolytic enzymes resulted in the destruction of the basic properties of the amino groups and the solutions became acidic in proportion to

the amounts of amino acid present. The titrations with N/100 NaOH were then continued and thus the amounts of " formaldehyde acid " present were estimated. Graphs I and II show the amounts of " free " and " formaldehyde acid " formed during peptic and tryptic digestion of albumin. It can be seen that the acid production was quite different in the two cases, " formaldehyde acid " steadily increasing above the amounts of " free acid " found in tryptic digestion, whereas in peptic digestion, the amounts of " formaldehyde acid " produced were always less than the amounts of " free acid " present.

The digestion of albumin by *A. lumbricoides* (buffered at *p*H 6·2) and *S. edentatus* (buffered at *p*H 7·1) was examined in the above manner. Graphs III and IV show the relative production of " free acid " and " formaldehyde acid " during the periods of digestion. It can be seen that both parasite proteases were tryptic in nature.

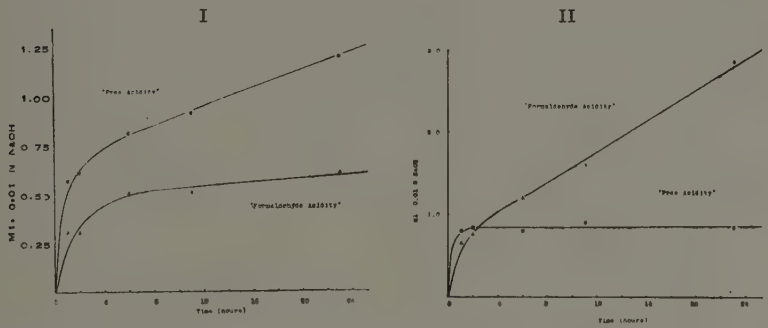
Effects of Hydrogen Ion Concentration on Protein Digestion.

A. lumbricoides.—The digestion of gelatin, blood-albumin and casein was tested at *p*H 5·3, 6·2, 7·1 and 8·0 (Sørensen's phosphate buffers) and about 9·4 (bicarbonate-phosphate mixture) by the formol titration method. Tubes containing 0·5 ml. of a 4% solution of the appropriate protein, 0·5 ml. of buffer, 0·2 ml. of enzyme extract and a drop of toluene were incubated for 6 days at 37°C. Altogether 8 experiments of this type were carried out and, with the one exception when the largest amount of casein was digested at *p*H 5·3, maximum enzyme activity was obtained at *p*H 6·2. Some typical results are shown in Graphs V, VI and VII.

S. edentatus.—Similar experiments were carried out using *S. edentatus* enzyme extracts. Typical results are summarised in Graphs V, VI and VII. Of the 7 experiments carried out in the examination of *S. edentatus* protease, all except one showed maximum enzyme activity at *p*H 6·2. The exception gave greatest digestion (casein was the substrate) at *p*H 5·3.

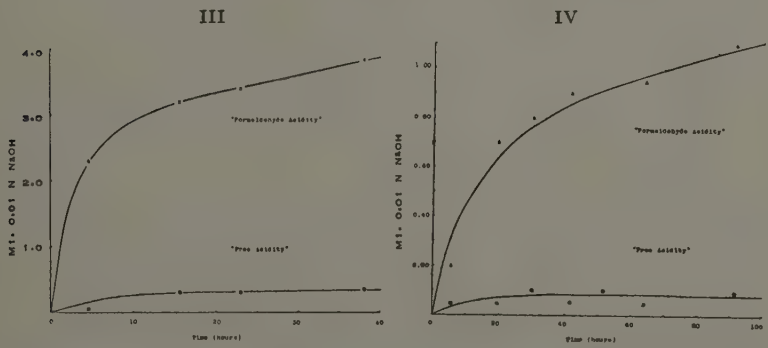
Results obtained at *p*H 9·4 when *S. edentatus* or *A. lumbricoides* enzymes were being examined must be regarded as approximate only. This is due to the consideration that heating would give rise to errors in the amino acid estimation and hence the complete titration of the $NaHCO_3$ was not attempted. Thus the initial end point to phenolphthalein in the " free acid " determination was taken without heating after which the " neutral

formalin " was added and the titration continued. It is not suggested that the quantities of " formaldehyde acid " estimated were very inaccurate but it is probable that the amounts recorded as being present were always slightly less than the true results.



GRAPH I.—Showing the amounts of " formaldehyde acid " and " free acid " produced by the action of mammalian pepsin on blood-albumin at *p*H 1·4.

GRAPH II.—Showing the amounts of " formaldehyde acid " and " free acid " produced by the action of mammalian trypsin on blood-albumin at *p*H 7·1.



GRAPH III.—Showing the amounts of " formaldehyde acid " and " free acid " produced by the action of *Ascaris lumbricoides* protease on blood-albumin at *p*H 6·2.

GRAPH IV.—Showing the amounts of " formaldehyde acid " and " free acid " produced by the action of *Strongylus edentatus* protease on blood-albumin at *p*H 7·1.

Relative Rates of Protease Action on Different Substrates.

The relative amounts of amino acid formed by the action of *S. edentatus* and *A. lumbricoides* proteases acting on substrates of gelatin, casein and albumin were examined at *p*H 6·2. In each experiment, three tubes were used, all containing the same amount of enzyme extract taken from the one source and the same amount of buffer but each tube having a different protein as substrate for enzyme action. After the addition of a little toluene to each tube, they were incubated for the same period, usually about 7 days, at 37°C. Amino acid was estimated as before.

Five such experiments were carried out. Table I shows the proportions of amino acids produced by protease action on gelatin, albumin and casein buffered at *p*H 6·2, the amounts produced from casein being taken as the standards for comparison.

Table I.

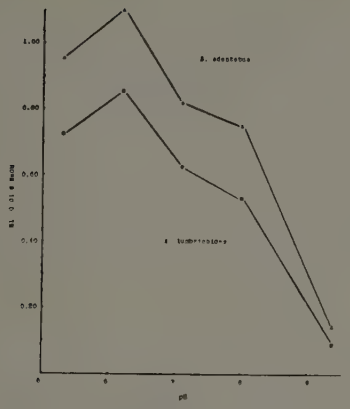
Showing the relative amounts of amino acid N produced by the hydrolysis of gelatin, blood-albumin and casein by *S. edentatus* and *A. lumbricoides* proteases.

Source of protease	Substrate for enzyme action.		
	Gelatin.	Albumin.	Casein.
A. lumbricoides ...	6·4	—	10
,, ...	6·3	4·9	10
,, ...	6·7	1·4	10
S. edentatus ...	0·9	0·7	10
,, ...	2·4	1·9	10

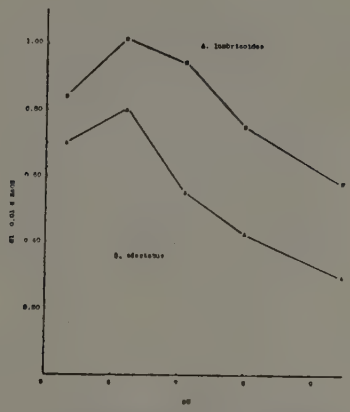
Relative Ability of *A. lumbricoides* and *S. edentatus* to Digest Proteins.

Enzymes were extracted from the intestines of *A. lumbricoides* and *S. edentatus* using the quantitative technique described by Rogers (1941). Tubes containing 0·2 ml. of enzyme extract, 0·5 ml. of buffer, a little toluene and 0·5 ml. of 4% solutions of gelatin, casein or blood-albumin, were incubated for 6 days at 37°C., after which amino acid production was estimated by the formol titration method. Owing to the inaccuracy of the determinations in the presence of bicarbonate, enzyme activity at

V



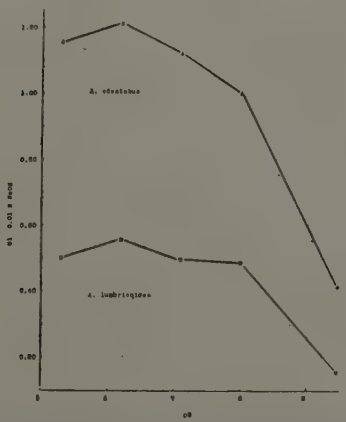
VI



GRAPH V.—Showing the amounts of 0·01 N NaOH necessary for the formol titration of the amino acids produced by *S. edentatus* and *A. lumbricoides* proteases acting on gelatin substrates at different hydrogen ion concentrations.

GRAPH VI.—Showing the amounts of 0·01 N NaOH necessary for the formol titration of the amino acids produced by *S. edentatus* and *A. lumbricoides* proteases acting on blood-albumin substrates at different hydrogen ion concentrations.

VII



GRAPH VII.—Showing the amounts of 0·01 N NaOH necessary for the formol titration of the amino acids produced by *S. edentatus* and *A. lumbricoides* proteases acting on casein substrates at different hydrogen ion concentrations.

about *p*H 9·4 was not investigated. Knowing the total weight of worms used to provide the proteases under examination and the volume of 50% glycerol required for extraction, it was possible to calculate the amounts of amino acid N formed by enzyme action per gram of worm. In Tables II, III and IV the amounts of amino acid N produced by parasite proteases per gram of worm in 6 days are compared. The " relative activity factors " shown in the tables were obtained by dividing the amounts of amino acid N produced by *S. edentatus* protease by the amounts formed by the action of *A. lumbricoides* protease and the figures therefore show the relative ability of the parasites to digest proteins.

TABLE II.

Showing the amounts of amino acid N produced from gelatin after 6 days incubation at 37°C. by proteases extracted from the intestines of 1 gram weights of worm tissue. For further explanation see text.

	*p*H	Mgs. of amino acid N produced from gelatin.		Relative activity factors
		A. lumbricoides	*S. edentatus.*	
No. 1.	5·3 ...	0·39	1·93	4·9
	6·2 ...	0·40	2·21	5·5
	7·1 ...	0·31	1·65	5·3
	8·0 ...	0·28	1·51	5·4
No. 2.	5·3 ...	0·23	1·70	7·4
	6·2 ...	0·24	1·98	8·3
	7·1 ...	0·20	1·21	6·0
	8·0 ...	0·17	0·97	5·7

TABLE III.

Showing the amounts of amino acid N produced from casein after 6 days incubation at 37°C. by proteases extracted from the intestines of 1 gram weights of worm tissue. For further explanation see text.

	*p*H	Mgs. of amino acid N produced from casein		Relative activity factors.
		A. lumbricoides.	*S. edentatus.*	
No. 1.	5·3 ...	0·64	20·33	31·8
	6·2 ...	0·64	24·32	38·0
	7·1 ...	0·55	22·52	40·9
	8·0 ...	0·54	20·10	37·2
No. 2.	5·3 ...	0·36	5·10	14·2
	6·2 ...	0·38	5·02	13·2
	7·1 ...	0·33	4·56	13·8
	8·0 ...	0·31	3·88	12·5

TABLE IV.

Showing the amounts of amino acid N produced from blood-albumin after 6 days incubation at 37°C. by proteases extracted from the intestines of 1 gram weights of worm tissue. Since the quantitative determination of amino acid formed by *A. lumbricoides* protease acting on an albumin substrate was only carried out in one series of experiments, it has been necessary to compare this set of results with those obtained from the study of two series of experiments using *S. edentatus* protease. For further explanation see test.

	*p*H	Mgs. of amino acid N produced from albumin		Relative activity factors
		A. lumbricoides	*S. edentatus*	
No. 1.	5·3 ...	0·30	0·87	2·9
	6·2 ...	0·38	0·96	2·5
	7·1 ...	0·21	0·65	3·1
	8·0 ...	0·20	0·53	2·6
No. 2.	5·3 ...	0·30	1·40	4·7
	6·2 ...	0·38	1·58	4·2
	7·1 ...	0·21	1·10	5·2
	8·0 ...	0·20	0·78	3·9

DIGESTION OF HAEMOGLOBIN.

Tubes containing 0·5 ml. of human blood diluted with water, 0·5 ml. of buffer and 0·2 ml. of enzyme extract were incubated at 37°C. At known intervals, samples were taken from the different tubes and examined spectroscopically. To obtain a true comparison of the strengths of the chromoproteins present in the different solutions, samples from the tubes were placed side by side in a glass cell which gave standard columns (about 0·5 mm. in height) of fluid for examination. A microspectrometer mounted on a microscope was used for spectroscopic examination. The cell containing the chromoproteins from the tubes was placed on the stage of the microscope and it was then possible, by moving the cell, to compare rapidly the strength of the absorption bands formed by the partly digested haemoglobin from the different tubes.

The presence of haematin in the digests was determined as follows. Tubes containing the digested haemoglobin were centrifuged strongly and the supernatant fluid pipetted off, after which the solid matter was washed with water and centrifuged. This procedure was repeated several times. Thus all the water soluble material was removed. The solid

matter was then dissolved in 0·5 ml. of warm NaOH and a little solid sodium hydrosulphite added. When haematin was present in the digested material its presence was indicated by globin-haemochromogen bands found on spectroscopic examination. Examinations for the presence of haematin in digested material were not carried out when *Strongylus* protease was being investigated as the enzyme extracts themselves contained haematin (Rogers, 1940c, showed that haematin was present in the intestine of *Strongylus* spp.). Table V shows the results obtained.

TABLE V.

Showing the rate of appearance and disappearance of reduced haemoglobin during the process of digestion of oxyhaemoglobin by *Ascaris* and *Strongylus* proteases. The strengths of the absorption bands were estimated visually (see text) and are indicated by the number of plus signs. Those signs between brackets refer to the strengths of globin-haemochromogen bands. Control tubes containing water instead of enzyme extract showed no change during the period of incubation.

Incubation period	*p*H	Strength of reduced Hb band	Colour of digest
Ascaris enzyme extract.			
3 hours	8·0	+	brownish-red
,, ...	7·1	+	light brown
,, ...	6·2	++	,, ,,
,, ...	5·3	+++	dark brown
7 hours	8·0	+	light brown
,, ...	7·1	++	,, ,,
,, ...	6·2	++	dark brown
,, ...	5·3	++	blackish-red
22 hours	8·0	+ (trace)	light brown
,, ...	7·1	+ (trace)	dark brown
,, ...	6·2	trace (+)	,, ,,
,, ...	5·3	absent (++)	blackish-red
Strongylus enzyme extract.			
1 hour	8·0	+	brownish-red
,, ...	7·1	++	,, ,,
,, ...	6·2	++	,, ,,
,, ...	5·3	++	,, ,,
5 hours	8·0	++	brown
,, ...	7·1	+++	,,
,, ...	6·2	+++	pinkish-brown
,, ...	5·3	+++	,, ,,
20 hours	8·0	+	brown
,, ...	7·1	++	greenish-brown
,, ...	6·2	++	,, ,,
,, ...	5·3	++	,, ,,

Discussion.

That the proteases secreted by *A. lumbricoides* and *S. edentatus* are tryptic in nature rather than peptic is not surprising in view of the fact that high HCl concentrations are necessary to activate the peptic type of enzyme. The situations of the parasites in their hosts and the nature of the materials they ingest (Rogers, 1940b and 1940c) suggest that considerable secretory activity on the part of the worms would be necessary to provide media suitable for peptic digestion, whereas it appears that optimum conditions for tryptic activity would occur during the digestion of a mixed diet. Both *Strongylus* (feeding on tissue and blood) and *Ascaris* (ingesting well mixed fluids from the hosts' small intestines) would include fats in their diets. The hydrolysis of these fats which would be taken in with fluids providing conditions suitable for rapid lipolytic action, would cause the formation of acids and thus give rise to a medium promoting protein digestion. Hence it appears that carbohydrate and fat digestion would proceed vigorously immediately after ingestion and proteolytic action, at first very slight, would become more rapid as fatty acids accumulated before absorption.

Since the parasite proteases digested albumin and gelatin most vigorously at *p*H 6·2 (casein possibly needed a slightly more acid media) these enzymes must differ widely from those of the parasites' hosts which would act best at about *p*H 8, the optimum reaction varying somewhat with different substrates. Actually the optimum hydrogen ion concentration for the action of parasite trypsin is outside the range for normal trypsin (there is a definite decrease in activity of mammalian trypsin at *p*H 6·5, Harrow and Sherwin, 1935). As in the case of some insects such as *Stenobothrus* and *Tettigonia* which have tryptic enzymes activated to the greatest extent at *p*H 6·2 (Schlottke, 1937) the properties of *A. lumbricoides* and *S. edentatus* proteases are exceptional. It is evident that Northrop's (1922) contention that the form and position of the *p*H-activity curve is determined solely by the titration curve of the substrate protein, is not supported by the results obtained in this investigation. In fact, Wigglesworth's (1928) suggestion that the *p*H-activity curve is determined, at least in part, by the nature of the enzyme, or by the nature of the associated substances, seems to apply in this instance.

No great difference between the nature of the proteases from the two parasites could be detected. However, it seems possible that, in relation

to its action on casein, *A. lumbricoides* protease was more efficient in splitting gelatin than *S. edentatus* (see Table I). As this point of difference between the parasites' enzymes is only of minor importance compared to the points of similarity, and as both proteases are remarkable in regard to their relationship to hydrogen ion concentration, it appears probable that the proteases found in the parasites would not be far removed from those present in their free-living ancestors.

It is apparent (see Tables II, III and IV) that *S. edentatus*, per gram of tissue, was far more active in digesting protein than *A. lumbricoides*. Acting on a gelatin substrate, *Strongylus* protease extracted from the intestine of a given weight of worm tissue, digested from 4·9 to 8·3 times as much protein as enzyme from a similar amount of *Ascaris* tissue. It was also found that *Strongylus* digested from 12·5 to 40·9 times as much casein and 2·5 to 5·2 times as much albumin as *Ascaris*. The variations in the figures giving the relative ability of the two parasites to digest proteins are probably due to: (1) slight differences in the periods the worms remained in the dead hosts before they could be collected and the enzymes extracted; (2) variations in the secretory activity in the different lots of parasites when they were removed from their hosts; and (3) minor changes in the technique of enzyme extraction, e.g. slight differences in the periods of trituration when the proteases were freed from the worm tissue.

However, in spite of the range of the "relative activity factors," it seems reasonable to consider *S. edentatus* to be highly efficient in the digestion of protein in comparison with *A. lumbricoides*. This is in accordance with the observations (Rogers, 1941) that the hydrolysis of fats was more rapid in the parasites of the horse than in those of the pig and adds further weight to the suggestion that the former worms feed on host tissue whereas the latter absorb partly digested material from the host gut lumen.

The processes of the digestion of haemoglobin in the two parasites can be determined from Table IV. Evidently the first change was the reduction of the oxyhaemoglobin, which proceeded most rapidly at the higher hydrogen ion concentration. Following this, there was a fall in the strength of the reduced haemoglobin bands and haematin was formed, at least in the case of *A. lumbricoides*. Rogers (1940b) has shown that haematin is plentiful in the posterior section of the intestine of *Strongylus*

and it seems probable that the digestion of haemoglobin in this parasite is similar to that in *Ascaris*. Briefly, then, the digestive enzymes which acted most vigorously at higher acidities, first reduced the oxyhaemoglobin and then split haematin from the resulting haemoglobin.

Rogers (unpublished) found that the haemoglobin in the body fluid of *S. edentatus* had a high affinity for oxygen and furthermore this body fluid was of such a high *p*H that the oxygen combining power would be further enhanced. These facts, together with the knowledge that oxygen is rapidly removed from ingested oxyhaemoglobin, lead to a possible explanation of why such large quantities of host blood are ingested by *Strongylus* (Rogers, 1940b) in spite of the fact that very large amounts of tissue are also engulfed (Rogers, 1940c). It seems possible that the oxyhaemoglobin is ingested partly to provide a source of oxygen and that this substance, after being freed from the oxyhaemoglobin in the gut, is passed through the intestinal wall to the body cavity where it combines with the parasite haemoglobin and so becomes available for assisting further metabolic processes.

Summary.

1. Tryptic-like enzymes have been extracted from the intestines and intestinal contents of *Ascaris lumbricoides* and *Strongylus edentatus*. The action of these enzymes, in relation to hydrogen ion concentration, has been examined.

2. The parasite trypsins resembled pancreatic trypsin in that the relative production of "free acid" and "formaldehyde acid" was similar, but though acting only on the alkaline side of the isoelectric points of the substrate proteins, optimum action on gelatin, blood-albumin and casein was obtained at *p*H 6·2.

3. The amount of protease extracted from a given weight of *S. edentatus* tissue was far greater than that from a similar amount of *A. lumbricoides*. Thus *Strongylus* digested 4·9 to 8·3 times as much gelatin, 12·5 to 40·9 times as much casein and 2·5 to 5·2 times as much blood-albumin as *Ascaris*.

4. Spectroscopic examination of the process of digestion of oxyhaemoglobin showed, first, the formation of reduced haemoglobin and then the

formation of haematin. These changes took place most rapidly at higher hydrogen ion concentrations.

ACKNOWLEDGMENTS.

Thanks are due to Mrs. L. E. Rogers, M.Sc., who assisted in the preparation of enzyme extracts.

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Dilution Egg-Counts and the Poisson Series.

By B. G. Peters, M.Sc., Ph.D.

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Researches involving dilution egg-counts, recently carried out by members of the staff of this Institute, have shown that the error variance, i.e. that between parallel counts, approximates more or less closely to the mean count. The following examples illustrate this point :—

	Sum.	n.	Mean.	Sum of Squares	n^1.	Mean Square.
A. ...	9,879	972	**10·1636**	6,495·67	648	**10·0242**
B. ...	2,093	216	**9·6898**	1,564·67	144	**10·8657**
C. ...	2,435	324	**7·5154**	1,660·67	216	**7·6883**
	14,407	1,512	**9·5284**	9,721·00	1,008	**9·6438**

(n^1 is the number of degrees of freedom available for error ; the data are taken from : (A) Peters, J. Leiper, & Clapham, 1941, *J. Helminth, 19* (1/2), 9–24 ; (B) J. Leiper, Peters, & Clapham, 1941, *J. Helminth, 19* (1/2), 25–34 ; (C) unpublished.)

The counts were all made by the same technique, and it is fair to total them in order to observe the average effect. This agreement between variance (or mean square) and mean is a feature of the Poisson distribution, and it has been assumed that dilution counts are in fact distributed according to the terms of that series, in all cases where the technique is adequate. Reasonable agreement between mean and error variance becomes, indeed, a criterion of the accuracy of the sampling technique. The purpose of the present note is to enquire a little further into the theoretical basis of this assumption, doubts having arisen as to whether high counts (say, up to 100) can be expected to conform to the Poisson series.

The essence of the McMaster (Gordon & Whitlock) technique is that one counts the eggs lying under a centimetre square engraved on a coverglass which is supported 0·15 cm. above a slide, i.e. the eggs contained in 0·15 cc. of faecal suspension. Since the suspending liquid is a half-saturated solution of salt, the eggs float up into one optical plane immediately below the coverglass, this making for ease in counting.

Thus, in effect, within the area of 1 cm.^{2} one counts a number of small areas which are the optical projections of helminth eggs.

However, the ratio of small areas to large area does not represent the density of eggs in the suspension, this being given by the ratio of the respective volumes. The volumes of helminth eggs vary according to the species, but most nematode eggs fall within the limits 50 to 90μ long by 20 to 45μ in diameter, with Nematodirus (at about 200$\mu \times 95\mu$) as the chief exception. Taking the average of the mean egg size for 7 common sheep nematodes, including Nematodirus, at 90$\mu \times 45\mu$, and regarding the eggs as square prisms of these dimensions, the average volume works out at about 0·0002 mm.^{3} The volume of suspension included under the square is 150 mm.^{3} and so there is room for 750,000 eggs closely packed in the available space.

Suppose the 0·15 cc. of suspension to consist of 750,000 unit volumes, and suppose further that 100 eggs are included in it (this would be a very large count in our experience). If these eggs are distributed at random, then there will be a very small but constant probability that any given unit volume will prove to be an egg, and a large probability amounting almost to certainty that it will not. Clearly, the binomial distribution will characterize successive counts, and the only question at issue is whether Poisson's limit to the binomial will be appropriate in those cases where many eggs are present. In the assumed case of 100 eggs, the probability, p, that any unit volume will be an egg is 100/750,000, $=$·00013; and the probability, q, that it will not be an egg is $(1-p)$, $=$·99987. The counts from successive slides with the same mean of 100 will be distributed according to the expanded terms of the binomial: $(p+q)^n$, where $n=750{,}000$. The mean of such a series is np, $=100$, and the variance is npq, $=99{\cdot}987$.

For a mean count of 1 the corresponding variance would be 0·9999987. Thus, the lower the mean the more closely does the variance approximate to it, n being constant. In other words, np approximates to npq as q approximates to unity. The Poisson series is simply the binomial series pushed to the limit where p is indefinitely small, q is unity, and n is so large that the mean, np, is an appreciable quantity. On the assumptions of the last paragraph, egg-counts with a mean of 100 should have a variance of 100 on Poisson theory, and 99·987 on binomial theory. The discrepancy is obviously negligible. Even with a mean of 1,000, which is beyond the limits of practical experience, the binomial variance is 998·7, a discrepancy of only 0·13 per cent.

One may be concerned only with the large eggs of Nematodirus, the mean egg size of which may be taken as $200\mu \times 100\mu$—a generous estimate. There will now be only 75,000 units to the large volume, and counts with a mean of 100 will have a binomial variance of 99·87, or relatively the same as that of 1,000 mixed eggs. Even here, then, the Poisson series will be quite appropriate.

It is known that counts of red cells or yeast cells in a haemacytometer conform to the Poisson series. But, compared with a red cell of about 8μ in diameter, a worm egg is so enormous that I was led to doubt whether it was justifiable to assume Poisson theory. To take an absurd case: if only 20 eggs could be packed solid in the available space, counts with a mean of 10 would have a true (binomial) variance of 5, or only half the Poisson value. But although an egg is so much larger than a red cell, the volume of liquid is also much greater in a McMaster slide, and the mean count much lower. Thus theory, supported by our own findings, shows that the Poisson distribution fits the facts.

The Effect of Mixing.

The preceding discussion assumes that eggs are distributed at random throughout the faecal emulsion. In order to ensure that they are so distributed, very thorough mixing is necessary, especially as the salt solution tends to float the eggs rapidly. From conversation with other workers in this field it would appear that many of them expect prolonged mixing to give an *even* distribution of eggs rather than a random one, in which case the variance would tend towards zero as the technique became more nearly perfect. This is surely in accordance with neither theory nor experience. When a pack of cards is thoroughly shuffled, one does not expect every fourth card to be (say) a club: one merely expects that any one card will have a 25 per cent. chance of being a club. And in counting eggs one does not find the (say) 16 eggs on a slide neatly arranged in four rows of four; more frequently one is struck by the unevenness of their distribution, as in a recent case where the three eggs of one slide were all found within a single microscopic field (there are about 25 fields to the square). Suppose it to be possible to obtain a faecal emulsion with the contained eggs suspended symmetrically in it; then, if these critics are right, further mixing should tend not to disturb but rather to maintain the arrangement! If they are seeking technical improvements such that a series of counts with a mean of 15 will all be 15, then I fear they are wasting time: they will have done well if 10 per cent. of their counts are 15. (Poisson expectation is 10·2436 per cent.)

Personal Error.

With counts in the region of 5 it is difficult to miss an egg or to count one twice. Using a mechanical stage, one scans the square in a series of about 5 strips, depending on the optical equipment used. One can afford to allow a slight overlap between adjacent strips for if an egg occurs near the edge of the first strip one remembers it when one finds it again near the opposite edge of the next. But with counts in the region of 50 more skill is required to count each egg once and once only, and it is unsafe to admit any overlap. A number of counters counting the same slides will agree as to low counts, but with high counts there will be a slight discrepancy. This is a personal error over and above the Poisson "error," and may be either random or systematic, according to the person. For this reason it is better to keep the counts reasonably low and to count more slides, by varying the original dilution. Alternatively, if high counts are thought desirable, it would be valuable to have the square centimetre subdivided by further engraved lines into 2 mm. strips so that the width of a strip would come within a single field.

Size of Counting Cell.

Various modifications of the original McMaster technique have been made, e.g. by Blount (1941)* who suggests increasing the area of the counting cell fourfold: "This automatically reduces by half the errors due to the use of the pipette and the shaking of the bottle." A fallacy appears to be involved in Blount's arguments. A suspension giving a mean and variance of 10 in the small slide will give a mean and variance of 40 in the large one, so that the *variability* will be reduced by one half (from 31·6% to 15·8%), but the precision of one count in the large cell is no greater than four counts in the small one, or than one count at four times the concentration in the small one. Blount prints a table of counts (p. 72), expressed in eggs per gramme, to show how much less error there is with the large counting cell; but this is equivalent merely to showing that means of four counts are less variable than single counts, which was already known. If one is counting (say) 1,000 eggs from a sample it makes no difference whether they are counted in one large slide or in several small ones, except that the parallel small-cell counts provide an estimate of the error variance, which the single count does not do. It is a feature of the Poisson distribution that the precision of a count depends simply on the number counted.

* Blount, W. P. *J. R. Army vet. Corps*, **12** (2), 68–78.

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Single *versus* Multiple Doses of Phenothiazine in Lambs.

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Introduction.

The earlier work of this Institute on phenothiazine in lambs (Peters *et al*, 1941; J. Leiper *et al*, 1941) using egg-counts, worm-counts, and lamb-weights as criteria of anthelmintic efficacy, has revealed conflicting results as between one criterion and another. Thus, the lowest dosage rate used (0·15 gm. per Kg.) led to a reduction in the egg-count, whilst even the highest rate (1 gm. per Kg.) led to no reduction in the worm-count: by an irony of chance the worm-counts were lowest in the untreated controls in both experiments. In the first experiment weights were not affected by the drug, and in the second the only significant effect was a set-back in those lambs given tablets. Numerous other workers have reported increased weights due to the drug, in long-term experiments; ours were too short in duration to show such increases, yet the danger of reinfestation precluded any extension of the time, the lambs being kept outside on infective pastures. The most obvious explanation of the egg- and worm-count results appears to be that the drug has a temporary inhibiting effect on egg-laying, which would presumably return to normal in a few weeks; but here again a prolonged experiment increases the liability to reinfestation.

These being the relevant circumstances, the present experiment was designed to take account of them in the following ways. In the first place the lambs were kept inside throughout the experiment, their stalls being cleaned out thoroughly twice a week to obviate reinfestation. Secondly, eggs were counted and lambs weighed three times in the week before treatment, three times immediately after treatment, and again three times a month later. The experiment would have been prolonged for a further month but for the death of one of the lambs, when it was terminated so that all worm-counts should be comparable. This arrangement enabled us to see if the fall in the egg-count was a temporary feature, and also whether lamb-weights were increased at the end of about five weeks. Thirdly, tablets alone were used because we had found them easier to administer than a drench: we now know

that this was because a large dose of bile salt made the drench unpalatable; a tasteless drench is far easier to give than tablets. This made it more convenient to relate the dose to the lamb rather than to its weight, thus avoiding the use of fractions of a tablet. In toxicological experiments there is good reason for relating dose to weight, but in the case of an anthelmintic passing through the intestine it remains to be shown whether, within wide limits, the diameter of the intestine is closely correlated with the animal's weight. Fourthly, the dose was considerably increased to 40 gm. per lamb, because we were anxious to secure significant reductions in the worm-counts; from the pre-treatment lamb-weights this gave an average of 1·75 gm. per Kg. Finally, we were interested in the possibility that a low concentration of drug maintained over a period of about 12 days might be more effective than the same total quantity given in one dose.

The tablets used in the previous experiment were unsatisfactory in that they did not readily break down, possibly owing to age. On this occasion we should like to thank Messrs. Cooper, McDougall & Robertson, Ltd., for providing without charge an ample supply of freshly compressed tablets. These contained the same weight of Phenothiazine (5 gm.) and were of approximately the same shape as the brand previously used, but they readily broke down in water and proved as satisfactory in use as such a large tablet can be expected to be.

Experimental design and statistical treatment were as in the last experiment (J. Leiper *et al.*, 1941). From an outside farm 15 lambs were brought in on the 12th May, 1941, kept inside throughout the experiment, and fed on a clover-mixture hay; they were sheared during the following fortnight. Faeces were collected from the rectum for egg-counts and lambs were weighed on the following dates: May 26, 28, 30; June 23, 25, 27; July 21, 23, 25. One lamb with a very high egg-count (a stray from another flock) and two with exceptionally low counts were excluded. The remaining 12 were divided at random into three groups of four: A, controls, B, given multiple doses totalling 40 gm. per lamb, and C, given each a single dose of 40 gm. Group B received 15 gm. on the 9th June and 5 gm. on the 11th, 13th, 15th, 17th and 19th, when group C was also dosed. Thus, the first post-treatment egg-counts and weighings occurred on the 4th to 8th days after completion of dosing, and the second series on the 32nd to 36th days after. One lamb from group B died on the 8th August, and the remainder were therefore slaughtered during the following week.

Results.

Table I gives the total egg-counts (sums of 9 counts) and total lamb-weights (sums of 3 weighings in ¼lb. units: divide by 12 to give lb.) for each lamb in each of the three treatment periods: X, before treatment; Y, immediately after treatment; and Z, five weeks after treatment. At the foot of each column the totals should be divided by 36 in the case of egg-counts and 48 in the case of weights to give the mean values per lamb.

Table I.

Total Egg–Counts (bold type) and Lamb-Weights (for explanation see text).

A. Controls.			B. Multiple.			C. Single.		
X	Y	Z	X	Y	Z	X	Y	Z
67	**117**	**150**	**58**	**6**	**9**	**168**	**14**	**27**
669	711	668	744	751	787	636	691	726
110	**162**	**169**	**89**	**24**	**22**	**109**	**1**	**2**
594	669	638	666	689	758	632	628	705
76	**88**	**129**	**88**	**0**	**0**	**72**	**5**	**6**
562	567	557	527	574	594	569	573	592
29	**108**	**151**	**130**	**21**	**33**	**147**	**15**	**33**
562	602	612	549	537	539	568	552	545
282	**475**	**599**	**365**	**51**	**64**	**496**	**35**	**68**
2387	2549	2475	2486	2551	2678	2405	2444	2568

Egg-Counts.

Analysis of covariance gave the coefficients of regression, b, the adjusted totals for each treatment group, and the standard errors, S.E.,

shown in the upper half of Table II. The column Y-*b*X takes into account the regression of the first post-treatment counts on the pre-treatment counts. It shows a marked increase in the control group and slight decreases in the two treated groups; the decrease appears greatest in group C (single dose) but the difference between B and C is not significant. The column Z-*b*X covers the regression of the second post-treatment counts on the pre-treatment counts. It shows a still greater increase in the controls; the decreases in the two treated groups

TABLE II.

Adjusted Totals, found by Covariance, of Egg-Counts and Lamb-Weights for Treatment Groups.

Group.	Y-*b*X	Z-*b*X	Z-*b*Y
Egg-Counts—			
A. Controls...	394·5	521·2	295·1
B. Multiple...	−53·2	−36·7	31·4
C. Single	−106·6	−68·8	45·6
b...	0·2855	0·2758	0·6399
S.E.	35·7	25·2	26·7
Lamb-Weights—			
A. Controls...	64·37	−321	−364
B. Multiple...	−36·7	−234	−163
C. Single	−59·4	−249	−154
b	1·0409	1·1715	1·1137
S.E.	59·9	86·0	60·0

are of the same order as before and again do not differ significantly one from the other. The column Z-*b*Y is intended to ascertain whether there has been a significant change in the final counts as compared with the first post-treatment counts; the treated groups now show increases but they are still significantly lower than the controls. Thus, there is a marked fall in the egg-count immediately after dosing, and this is largely (but not wholly) maintained for at least a month after

dosing. Moreover, there is no significant difference between multiple and single dosing; so far as it goes, the evidence leans in favour of single dosing.

Lamb-Weights.

The lower half of Table II deals similarly with lamb-weights, though in this case the treatment variance was not significantly greater than the error variance. It follows that the weight data are not conclusive, and can only be considered as suggestive. The first column indicates that the treated groups have lost weight slightly, whilst the controls have slightly gained. The second column, adjusting final by pre-treatment weights, shows a fall in all three groups; and the third, adjusting final by first post-treatment weights, confirms this. In this third case the treatment variance almost achieves significance and justifies the application of the method outlined in the postscript to Peters *et al.*, 1941, p. 24, except that in this case the controls were compared with the mean of groups B and C. As a result it was shown that the loss of weight in the treated groups was significantly less than that in the controls.

Thus, there is a suggestion that phenothiazine at 40 gm. per lamb leads to a temporary loss of weight during the week after dosing, and there is definite evidence that this loss is at least made good during the following month.

Worm-Counts.

For the first time, we can report significant reductions in the post-mortem counts of several genera of nematodes, as judged by comparing the counts of the treated lambs with those of the controls. The test of significance was whether or not the variance between the three groups was significantly greater than that between the four replicate lambs in each group. Where this test just failed, the controls were compared with the more promising of the treated groups.

Complete counts, summarized in Table III, were made of the following genera: Bunostomum, Haemonchus, Chabertia, Oesophagostomum, and Trichuris. Counts were estimated by sampling not more than 1/25th of the whole contents in the case of the genera: Strongyloides, Nematodirus, Ostertagia, and Trichostrongylus. Abomasal and intestinal contents had been run together, and the males of Trichostrongylus were therefore further differentiated into species so as to provide separate data for *T. axei* from the abomasum. The genera Cooperia and Capillaria were found in only one lamb each,

and are therefore not considered further. Moniezia occurred in five of the 12 lambs but, being most numerous in the treated groups, is omitted from the table. The latter gives under each species the variance ratio, i.e., the ratio of the treatment-group variance (with 2 degrees of freedom) to the error variance (with 9 degrees). The critical tabulated values of this ratio, which should be exceeded if significance is to be claimed, are: at the 5 per cent. point, 4·26; at the 1 per cent. point, 8·02. Values less than unity have no statistical

TABLE III.

Total Worm-Counts for each Treatment Group of 4 Lambs (for explanation see text).

	Group: A	B	C	Total of 12.	Variance Ratio	*t*
Oesophagostomum ...	5	3	1	9	0·631	—
Chabertia	204	11	2	217	10·973*	—
Trichuris	106	88	115	309	0·081	—
Bunostomum	15	18	7	40	0·904	—
Haemonchus	813	28	5	846	5·266*	—
Ostertagia	8333	6227	1225	15,785	3·270	2·488*
Nematodirus	2957	1358	200	4,515	1·976	1·978*
Strongyloides	7267	1540	1625	10,432	3·799	2·371*
Trichostrongylus ...	38570	6085	1275	45,930	13·170*	—
Males only of *T. axei*	4950	325	100	5,375	7·363*	—
T. colubriformis ...	3550	1575	75	5,200	2·812	2·364*
T. vitrinus	825	300	75	1,200	3·344	2·520*

* Significant.

use: they can be taken as signifying no effect. It will be seen that a significantly lower worm-count in the treated groups occurs for the genera Chabertia, Trichostrongylus, and Haemonchus, and for the males of *T. axei*. Phenothiazine has definitely been effective against these parasites. In no case is there a significant difference between multiple and single dosing, but in every case the single dose gives an apparently better result. In the face of this cumulative evidence,

confirmed by the egg-counts, it must be judged that this is a real effect.

Of those genera which fail to pass this test of significance, the controls have been compared with the single-dose group, omitting the multiple-dose group. This has the effect of reducing the degrees of freedom for treatments to unity, and allows the *t*-test to be applied, where *t* is the square root of the variance ratio. The table also gives the values for *t* in these cases, the critical value being 1·833. This enables us to claim a real, if less marked, effect against Ostertagia, Nematodirus, Strongyloides, and the males of *T. colubriformis* and *T. vitrinus*. This result is interesting since both Nematodirus and Strongyloides have been generally considered as unaffected by phenothiazine.

There remain Oesophagostomum and Bunostomum, neither of which shows any significant reduction in numbers. This may mean that they have not been affected by the drug or, at least in the case of Oesophagostomum, that they were present in too small a number and in too few of the lambs for the effect to be revealed.

Discussion.

Since in previous experiments worm-counts did not confirm the effects of phenothiazine indicated by egg-counts, it was thought that the drug might have a temporary depressing action on egg-laying. This is not borne out by the present experiment, where the reduced egg-counts are largely, but not wholly, maintained for five weeks after dosing. On the other hand, there has in this case been a real reduction in the worm burden and this is obviously the main cause of the egg-count reduction, especially when one remembers the marked reduction in that large and prolific worm Haemonchus. Re-examination of the data of the last two experiments shows that, although total worms were more numerous in the treated troups, Haemonchus was less numerous, though not significantly so by the ordinary tests of significance. It is quite possible that the true explanation of the anomalous results previously obtained is as follows: (a) Haemonchus responds to small doses of phenothiazine; (b) Haemonchus being a prolific species, this leads to a significant reduction in the egg-count; (c) owing to the exceptionally high variation among replicate worm-counts, the real reduction in the Haemonchus-count fails to achieve significance.

It is clear that a dose of 40 gm. per lamb, given as a single dose rather than factionally, leads to a very marked reduction in the stomach worms Haemonchus and *Trichostrongylus axei*, and to a less marked reduction in Ostertagia. The efficiency of phenothiazine in cases of parasitic gastritis, claimed by Taylor, is thus substantiated, but at a dose level much higher than he recommends. The drug is also very effective at this rate against Chabertia, which again confirms the findings of other workers. It has some effect, though less marked, against the trichostrongylids of the small intestine and (surprisingly) against Strongyloides. It is apparently useless at this rate against Bunostomum and Moniczia in the small intestine, and Trichuris in the large. We have no evidence respecting Cooperia and Capillaria, and insufficient evidence respecting Oesophagostomum.

These real reductions in the worm burden, however, were not accompanied by any significant increase in weight over a period of five weeks. Indeed, there appears to have been an immediate set-back in the treated groups, subsequently made good. Possibly weights of treated groups would have improved later, if the lambs had been kept alive. Certainly they were kept under artificial conditions, for purposes of experimental control, and were on a poor diet, but even so the controls might have been expected to lose weight more rapidly than the treated groups. And, under natural conditions on infective pastures, they could have acquired a considerable reinfestation in five weeks. Again, these lambs were not clinical cases of parasitic gastritis or of any other helminthiasis, and in practice so large a dose would presumably not be given merely as a prophylactic. Nevertheless, it must be pointed out that the limited results of this treatment would have cost the sheep farmer about 1s. 9d. per lamb for the bare cost of the tablets, and it seems at least doubtful whether this would have been an economic procedure. In other words, our view is that phenathiazine at its present price and in doses sufficient to effect a marked reduction in worm-counts is not an economically satisfactory anthelmintic for prophylaxis. Whether or not it is satisfactory in the therapy of clinical helminthiasis is a question demanding further statistically controlled experimentation.

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The Effect of Phenothiazine on the Weights of Worm-free Sheep and Goats.

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In the spate of recent papers on the use of phenothiazine as an anthelmintic are many which claim an increased weight in treated animals as compared with controls. So far as we are aware, the presence of worms in the treated animals is at least assumed in all these cases, though it is true that in some no evidence of infestation is given. In other words, the increased weight is presumed to be the direct result of removing the worms. If this presumption is justified it follows that weight changes can validly be used as a criterion of anthelmintic efficacy, a criterion which is valuable for at least two reasons, one practical and the other somewhat abstruse but none the less important. The practical reason is that an increase in weight is usually the ultimate objective of treatment: the removal of worms is largely an academic process unless it leads to improved general condition.

The more abstruse reason is concerned with precision in the statistical estimation of significance in a properly planned experiment. Of the three criteria recently used by this and other institutes, worm-counts, egg-counts and weights, worm-counts suffer from two main disadvantages affecting precision: first, the variation among animals of the same flock is very high, which means that in order to reveal significance either there must be several replicates treated alike or the treatment differences must be very marked; secondly, there is for most worm species no means of knowing how many worms were present before treatment, from which it follows that the covariance method of analysis cannot be used and one is reduced to comparing treated animals with controls. This objection does not apply, of course, to the elaborate "Critical Test," which counts also worms passed in the faeces as the result of treatment, nor wholly to worms like Strongyloides and Nematodirus with identifiable eggs capable of giving some measure of infestation before treatment. In contrast to worm-counts, egg-counts and weights both lend themselves to periodical estimation before and after treatment, and therefore to statistical analysis by the covariance method. The essence of this method is

to determine the regression of post-treatment values, y, on pre-treatment values, x, in respect of the " Error " component in analysis. Then, instead of dealing with the post-treatment values as such, one deals with the set of adjusted values, $(y\text{-}bx)$, where b is the regression coefficient. Clearly, these adjusted values should tend towards zero, allowing for random variation, unless treatment has had a real effect. The method is obviously a most sensitive one.

In determining the regression coefficient one of the degrees of freedom allotted to error is sacrificed. Thus, if ordinary analysis of y^2 gives an error sum of squares with n degrees of freedom, this sum can itself be analysed into two components: (i) the adjusted error squares $(y\text{-}bx)^2$, with $(n\text{-}1)$ degrees of freedom, and (ii) regression, with one degree. The usual variance-ratio test can then be applied to find whether or not regression has removed a significantly large proportion of the original error. Examples of this analysis of residual error are to be found in our previous papers (e.g., Peters *et al,* 1941, Table VI, p. 17). Our experience with a considerable number of these analyses is that, in the case of weights, regression always accounts for a large proportion of the original error, so that the error component in the analysis of $(y\text{-}bx)^2$ is conveniently small and the test sensitive. On the other hand, in the case of egg-counts, the proportion of error ascribable to regression is usually small and often not significant, so that the test is in comparison not so sensitive. On this score, weights are preferable to egg-counts.

The above digression was to show why weights would be a valuable criterion of anthelmintic efficacy, if the weight increases of treated animals were due to loss of worms. For it is at least possible that such increases are due to some bactericidal or merely roborant effect of the drug, in which case it might still be advisable to give animals phenothiazine but weight increases could not be justifiably used as a criterion of anthelmintic efficacy.

We have therefore tested phenothiazine on a number of sheep and goats reared indoors since birth and known to be free from worms. For this purpose 6 kids and 6 one-year-old goats, 6 lambs and 6 one-year-old sheep were divided at random into two groups of three in each case, the members of one group receiving 15 gm. of phenothiazine in 5 gm. tablets (kindly supplied by Messrs. Cooper, McDougall & Robertson, Ltd.) on the 4/7/41. These animals and the controls had been weighed on the 30/6, 2/7, and 4/7/41. The three treated kids died on the 9/7, 5/8, and 17/8/41, respectively 5, 32, and

44 days after treatment, but no obvious cause of death could be found and the only macroscopic lesions were small petechial haemorrhages on the outer surface of the myocardium. This entailed discarding the group of 6 kids from the experiment.

TABLE.

Weights of Animals in Pre-treatment (X) and in 1st (Y) and 2nd (Z) Post-treatment periods.

(Each value is the sum of 3 weights in ¼ lb. units : divided by 12 to give lb.).

		X	Y	Z
Goats.	Controls	591	531	463
		560	547	538
		534	533	567
	Treated	672	638	622
		582	569	565
		653	669	671
Lambs.	Controls	744	670	737
		722	661	738
		553	505	553
	Treated	679	663	703
		752	747	752
		485	446	—
Sheep.	Controls	951	944	995
		951	935	994
		916	919	954
	Treated	821	826	871
		803	810	837
		673	627	646

The remaining animals were again weighed on the 11/8, 18/8, and 22/8/41, respectively 38, 45, and 49 days after dosing. Analysis of covariance of these and the pre-treatment weights showed that the

treatment variance was not significantly greater than the Treatment-by-Host interaction variance, nor than the error variance. There had been a general loss of weight, less marked among the treated animals but not significantly so. Nevertheless, the treatment variance did not fall far short of significance. Equating the variance ratio, Treatment/Error, with t^2 gave $t = 1{\cdot}708$ compared with the critical 5 per cent. value of 1·796. It was therefore felt desirable to repeat the treatment, giving this time 5 gm. to each treated animal on each of the dates 22/8, 5/9, and 19/9/41. Meanwhile, one of the treated lambs died on the 2/9/41, compelling us to make use of the so-called "Missing Plot" technique in the analysis of the final weighings, which were determined on the 13, 15, and 17/10/41. Analysis of covariance of these and the pre-treatment weights gave a treatment variance for $(z\text{-}bx)^2$ much lower than either the interaction or error variances, though within the limits of random variation.

It is therefore reasonable to conclude that, under the conditions of the experiment, phenothiazine had no effect on the weights of worm-free sheep and goats over a period of 15 weeks. The experimental conditions were necessarily artificial in the case of animals reared indoors on an abnormal diet, but there seems no good reason why, if phenothiazine had a beneficial action apart from its anthelmintic effect, that action should not have been revealed. We therefore take it that weight improvements in treated animals are likely to be due to the removal of worms and that weight data can validly be used as a criterion of anthelmintic efficacy.

Summary.

1. A total dose of 30 gm. of phenothiazine, given as tablets in one dose of 15 gm. and three of 5 gm. to three goats and six sheep known to be worm-free, had no effect on the weights of the animals in comparison with equal numbers of worm-free controls, over a period of 15 weeks from the first dose.

2. It is concluded that weights can validly be used as a criterion of anthelmintic efficacy; they not only constitute an obvious economic criterion, but also are statistically more sensitive than egg-counts when subjected to analysis of covariance.

Reference.

Peters, B. G., Leiper, J. W. G. & Clapham, P. A. 1941.—"A phenothiazine experiment statistically treated." *J. Helminth.* **19** (1/2), 9–24. (*W.L.* 11224b.)

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The Differentiation of *Coenurus* species by Hook Measurements.

By P. A. Clapham, D.Sc. and B. G. Peters, M.Sc., Ph.D.

(*From the Institute of Agricultural Parasitology, St. Albans.*)

Introduction.

During the intervals which have inevitably occurred in more urgent tasks, we have considered the problem of differentiating species of *Coenurus* on the basis of various hook dimensions, giving due weight to the variation that necessarily occurs in all biological data of this kind. The scolex of the taeniid cestodes is usually armed with a double crown of hooks, the large hooks of one circle alternating with the small hooks of the other. On any one scolex both circles have the same number of hooks, and this number is to some degree characteristic of the species but at the same time is liable to slight variation. The taeniid hook has a characteristic shape (Fig. 1) which has been likened to that of a jack-knife, showing a blade, guard and handle, the guard and handle being the roots of the hook embedded in the rostellum. Numerous dimensions, more or less clearly defined, are possible with a hook of this shape.

Prof. R. T. Leiper kindly placed at our disposal a considerable amount of larval and adult material of the genus *Multiceps,* the larval form of which is a coenurus—a cyst having invaginated within it several scolices.

This paper is concerned with cysts and adults of *M. glomeratus* and *M. serialis,* being experimental material of which the genetic history was known. These two species have the same number of hooks on the rostellum, and the hooks are very similar in both shape and size. Seven different dimensions were measured on the large hooks and six on the small. On the basis of these dimensions we attempted to answer the following questions.

1. What differences are to be found in the shape and size of larval as compared with adult hooks?

2. What differences are to be found between the available material of *M. glomeratus* and *M. serialis*?

3. What dimensions are the most satisfactory as judged by the two main criteria: (a) of giving the most highly significant differences under questions 1 and 2: and (b) of having a low inherent variability?

The material has been handled, camera lucida drawings made and dimensions measured by P. A. C. The data have been statistically treated by B. G. P., who is also responsible for presentation.

Material.

The *M. glomeratus* material came orginally from a natural infestation in a wild London mouse. This material was fed to a dog, and eggs from the resulting adults to a gerbille. Cysts from the gerbille were fed to another dog, and eggs from the resulting adults to a rabbit in which two coenuri developed. One was used for hook measurements (" Cyst 1 ") and the other fed to a dog. Some of the resulting adults were used for hook measurements and eggs from them were fed to rabbits; one of the cysts produced was used for hook measurements (" Cyst 2 "). Thus, the " Cyst 1 " and " Adult " material are from the same generation, and the " Cyst 2 " material from the next generation.

The *M. serialis* material came originally from two naturally infested wild rabbits, obviously litter mates, each of which harboured one cyst. One cyst was divided into two; part was used for hook measurements (" Cyst 1 ") and part fed to a dog. The resulting adults were used for hook measurements. The other naturally acquired cyst was used wholly for hook measurements. Thus, the " Cyst 1 " and " Adult " material are from the same generation and it is probable (but not certain) that the " Cyst 2 " material was also from this same source.

Technique.

All material was fixed in 10% formol saline within a short time of removal from the host, and was preserved in glycerine-alcohol (5% glycerine in 70% alcohol). Hooks were drawn by camera lucida and dimensions measured from the drawings.

Nothing was known *a priori* about the way in which variation might occur, in respect of any one dimension. We had in mind the possibility of significant variation between species, and between the stages (cysts and adults) of any one species. But, apart from this, in the case of cysts, there might prove to be significant variation from one cyst to another within a species, from one group of scolices to another within

a cyst, and from one scolex to another within a group. It was therefore essential to accumulate and analyse the data in such a way as to test these possibilities. The statistical procedure known as " Analysis of Variance " is appropriate to this purpose, and the method of calculation applicable to hooks from cysts is shown in Table 1. Here the total variance of all hooks from the general mean is analysed into its various components; variance between species, between cysts per species, between groups per cysts, between scolices per group and between the hooks of a single scolex. The latter is the so-called " error "

TABLE I.

Scheme for Analysis of Variance (Cystic Hooks).

Let x = Dimension of any one hook.
" X = total of 300 hooks for whole of data.
" S = " 150 " " each species.
" C = " 75 " " cyst.
" G = " 15 " " group of solices.
" H = " 5 " " scolex (head).

Then the analysis of variance will be as follows :

Source of Variation.	Sum of Squares.	Deg. of Freedom.
(*a*) Between species	$\Sigma(S^2)/150-X^2/300$	1
(*b*) Between Cysts per species	$\Sigma(C^2)/75-\Sigma(S^2)/150$	2
(*c*) Between Groups per cyst	$\Sigma(G^2)/15-\Sigma(C^2)/75$	16
(*d*) Between Scolices per group	$\Sigma(H^2)/5-\Sigma(G^2)/15$	40
(*e*) Within Hooks per scolex (Error) ...	$\Sigma(x^2)-\Sigma(H^2)/5$	240
(*f*) Total	$\Sigma(x^2)-X^2/300$	299

variance, the square root of which is the standard deviation of the dimension concerned. In each species two cysts were available. We decided to measure the hooks from five different groups in each cyst, and from three different scolices in each group. Finally five large and five small hooks were taken at random from each scolex. In this respect it is important to take hooks from different parts of the rostellum for, if an abnormally small (or large) hook is found, there is a tendency for adjacent hooks to show some degree of the same abnormality : in other words, abnormally sized hooks tend to be grouped close together.

Thus, from the coenuri, 300 large and 300 small hooks were measured altogether. In the large hooks 7 dimensions, and in the small hooks 6, were measured (as indicated in Fig. 1), so that the coenuri provided 3,900 measurements.

The analysis is simpler in the case of adults, since cysts and groups do not appear. Five large and five small hooks were measured from each of 15 scolices from each species, making a further 1,950 measurements, or 5,850 altogether.

TABLE II.

Analysis applied to dimension *BC* (Large Hooks, Cystic).
(For explanation, see Text).

Source.	Sum of Squares.	D. of F.	Variance	Variance Ratio.
(*a*) Species	458·8426	1	458·8426	(a/b) 1·94
(*b*) Cysts	473·7730	2	236·8865	(b/d) 3·36*
(*c*) Groups	564·3981	16	35·2749	——
(*d*) Scolices	2,819·7163	40	70·4929	(d/e) 3·35***
(*e*) Error	5,056	240	21·0667	[σ=4·5898]
(*f*) Total	9,372·73	299	31·3469	[V%=5·3663]

The method of using the analysis of variance can be illustrated from Table 2, which gives the analysis for the dimension *BC* in the case of large cystic hooks. The variance for Species (a) is greater than the next highest variance—in this case, Cysts (b), but not significantly greater, as can be ascertained by entering the table of variance ratios under the appropriate degrees of freedom (1 and 2 respectively, in this case). This means that observed differences between the two species, in this dimension, must be ascribed to the variation which occurs from one cyst to another within a species. The Cyst variance (b) is significantly greater than the next highest variance—in this case, Scolices (d), the variance ratio being larger than the tabulated value at the 5% point for this ratio but smaller than the 1% value. (Conventionally, ratios significant at the 5% point will be given a single asterisk, those at the 1% point two, and those significant at the 0·1% point three asterisks.) Hence there is a real variation from

one cyst to another (within a single species) over and above the differences which might be due to variation from one scolex to another. The Group variance (c) is actually lower than that for scolices (d), though not significantly so, so that none but random differences can be found between groups of scolices in any one cyst. The Scolex variance (d) is greater than the variance between hooks of a single scolex (Error variance, e) in a ratio which is significant even at the 0·1% points: there is therefore a very definite variation from scolex to scolex within a group. The standard deviation for the dimension *BC*, is in absolute units 4·5898μ or as a percentage of the mean, 5·3663%. The total variance (of all 300 hooks from the grand mean) is significantly greater than the error variance; this justifies the procedure of analysis of variance, which has eliminated from our estimate of error the two other sources of variation (between cysts and between scolices) shown to be significantly great.

The error variance can now be validly used for comparing cysts or scolices. Thus, the standard error of the differences of means between two cysts (for each of which there are 75 measurements) is taken as:

$$\sqrt{\frac{21{\cdot}0667\times 2}{75}} = 0{\cdot}7495\mu :$$

and that between two scolices (for each of which there are 5 measurements) as:

$$\sqrt{\frac{21{\cdot}0667\times 2}{5}} = 2{\cdot}9029\mu$$

The ratio of any such difference to its standard error gives the statistic *t*, the critical 5% value of which (for the 240 degrees of freedom on which the error variance is based) is 1·97. Thus, any difference which exceeds twice its standard error is probably real.

Data.

It is impracticable to publish the 5,850 values or even the detailed analysis of variance for each dimension; all the data are filed at the Institute of Agricultural Parasitology and we have room here for only the relevant statistics calculated from them. It will be convenient to deal with the three problems separately.

1. *Differences between larvae and adults.*

Table 3 gives the mean of each dimension for larval and adult hooks respectively, the difference, and the ratio of the difference to its

standard error (=t). In each case the cysts contribute 120 degrees of freedom and the adults 60 degrees, making 180 in all. The combined "Cyst+Adult" error variance is the sum of the two sums of squares for error, divided by 180, and the square root of this is the standard deviation (=σ). There are 150 cystic and 75 adult values so that the standard error of the difference is:

$$\text{S.E.} = \sigma\sqrt{\frac{150+75}{150\times 75}} = \frac{\sigma}{7{\cdot}0711}, \text{ and}$$

$$t = \frac{7{\cdot}0711\,(\bar{x}_1 - \bar{x}_2)}{\sigma}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the two means to be compared. The critical (5%) value of t is 1·98; all dimensions for which t exceeds this value show a real difference between cysts and adults. In the case of *M. glomeratus*

TABLE III.
Comparison of Cystic and Adult Hooks.

A. Multiceps glomeratus.

Hook	Dimension	Cystic Mean a	Adult Mean b	Difference (b−a)	$t=\frac{\text{Difference}}{\text{St. Error}}$
Large	AB	62·707	62·187	−0·520	1·306
,,	BC	86·767	86·067	−0·700	1·268
,,	AC	134·067	134·000	−0·067	0·108
,,	AE	50·240	49·533	−0·707	2·330*
,,	AD	91·640	91·600	−0·040	0·075
,,	ED	49·593	49·893	+0·300	0·764
,,	AF	64·027	64·627	+0·600	2·053*
Small	ab	43·460	43·427	−0·033	0·098
,,	bc	55·540	55·347	−0·193	0·393
,,	ac	84·280	81·920	−2·360	3·968*
,,	ae	37·087	37·053	−0·033	0·102
,,	ad	68·607	68·613	+0·007	0·013
,,	ed	36·220	37·480	+1·260	3·216*

B. Multiceps serialis.

Large	AB	63·107	65·267	+2·160	5·212*
,,	BC	84·293	88·320	+4·027	5·709*
,,	AC	136·167	140·907	+4·740	7·500*
,,	AE	55·740	57·253	+1·513	2·652*
,,	AD	93·720	105·693	+11·973	22·219*
,,	ED	51·293	54·693	+3·400	8·600*
,,	AF	69·486	78·746	+9·260	9·574*
Small	ab	47·207	50·987	+3·780	9·829*
,,	bc	57·793	61·147	+3·353	6·485*
,,	ac	90·940	96·733	+5·793	8·629*
,,	ae	43·093	44·520	+1·427	4·095*
,,	ad	73·480	80·213	+6·733	12·062*
,,	ed	42·793	40·787	−2·007	4·964*

this applies to the dimensions *AE, AF, ac, ed.* The curious feature here is that both *AE* and *ac* are smaller in the adult than in the cyst. Most of the non-significant differences are in the same sense. Inspection of the details show that hooks from " Cyst 2 " were larger than the present adult hooks and those from " Cyst 1 " were smaller; for most dimensions the mean of the two cysts was a little longer than that of the adults. This suggests that there is no considerable difference between cystic and adult hooks: i.e. that the hooks can already reach their full size in the coenurus stage.

In the case of *M. serialis* the adult hooks were significantly larger than the cystic hooks in all dimensions except *ed,* where they were significantly smaller. In comparison with *M. glomeratus* results, this suggests that the *M. serialis* cysts may not have been fully grown. It will be seen from Table 3 that the differences are in the region of 10%, and that the highest degree of significance attaches the dimensions *AD* and *ad.*

2. *Differences between* M. GLOMERATUS *and* M. SERIALIS.

Analysis of variance as shown in Table 1, applied to the various dimensions of the hooks, revealed the fact that the " Species " variance

was not significantly greater than the variance between the cysts of a single species, except in the case of the dimension *AE* (large hooks). In other words, *AE* is the only dimension by which the two species can be differentiated in their larval stage, on the basis of the present material. On the other hand, all the dimensions of both large and small hooks show a significantly large species variance in the case

Table IV.
Comparison of *M. glomeratus* and *M. serialis*.

Stage	Dimension	*M. glomeratus* Mean	*M. serialis* Mean	Difference of Means	$t=\frac{\text{Diff.}}{\text{S.E.}}$
Cyst	AE	50·24	55·74	5·50	12·782
Adult	AB	62·187	65·27	3·08	7·342
,,	BC	86·07	88·32	2·253	3·250
,,	AC	134	140·907	6·907	10·438
,,	AE	49·53	57·253	7·72	25·138
,,	AD	91·6	105·693	14·093	24·900
,,	ED	49·893	54·693	4·8	10·808
,,	AF	64·627	78·747	14·12	12·211
,,	ab	43·427	50·987	7·56	19·000
,,	bc	55·347	61·147	5·8	10·825
,,	ac	81·92	96·73	14·813	24·660
,,	ae	37·053	44·52	7·47	21·370
,,	ad	68·613	80·213	11·6	21·654
,,	ed	37·48	40·787	3·307	8·010

of adult hooks. Each pair of means, the absolute value of the difference, and its value relative to its standard error ($=t$), are shown in Table 4. It will be seen that *M. serialis* hooks are on the average consistently larger than those of *M. glomeratus*, though the ranges of individual measurements overlap.

3. *Suitability of Dimensions.*

From what precedes, it is clear that dimension *AE* is the only one available for comparing the two species in the larval stage. In addition, it is one of the four dimensions showing a significant difference between cysts and adults in *M. glomeratus*. In *M. serialis AD* shows by far the highest degree of significance in comparing cysts with adults. This suggests that *AD* and *AE* are likely to be the most useful dimensions to use in future. As will be seen from Fig. 1 these two dimensions measure respectively the longer and shorter sides of the hook blade; and it is in accordance with general experience that the blade shows less variability than the roots of the hook.

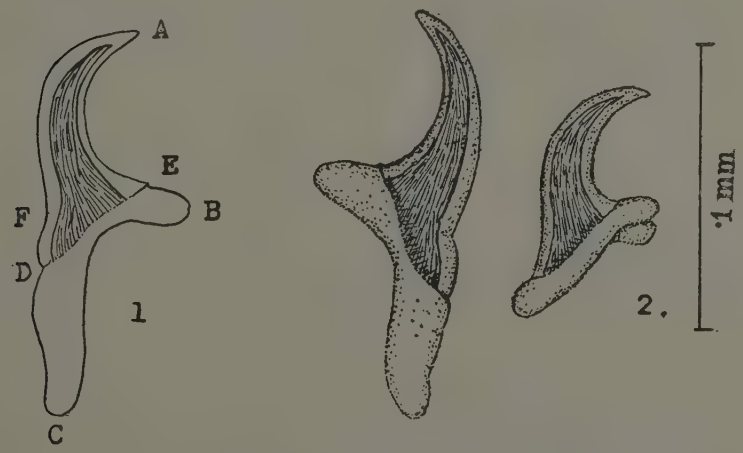

Fig. 1.—The typical taeniid hook.

Fig. 2.—Hooks of *M. glomeratus*.

Summary and Conclusions.

1. Hook dimensions of *Multiceps glomeratus* show that the hooks are capable of reaching full adult size in the coenurus stage.

2. The available material showed that *M. serialis* hooks were slightly larger than those of *M. glomeratus* in the adult stage; in the coenurus stage the differences were not significant.

3. Statistically, the dimensions measuring the length of the blade of the hook are the most satisfactory.

4. There are significantly large variances in most cystic hook dimensions from one cyst to another, and from one scolex to another within a single cyst, but not from one group of scolices to another. It follows that in biometric work it is important to sample several scolices from each cyst, and several cysts of any one species, but that groups can be disregarded.

5. The variability of most hook measurements is in the region of 5%; i.e. the standard deviation is about 5% of the mean dimension.

6. The danger of extensive generalisation from limited data should be guarded against. Thus, while the species variance was significantly larger than the cyst variance in the case of adult hooks in the material available, it is quite possible that this would no longer have been the case if material from other sources had been available.

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An English Case of *Coenurus cerebralis* in the human brain.

By Phyllis A. Clapham, D.Sc.

(*From the Institute of Agricultural Parasitology, St. Albans.*)

In the spring of 1941 a male patient died at Peterborough and the autopsy which followed showed that death was due to pontine haemorrhages. As the brain also carried a coenurus, it was submitted to me for further examination. I am much indebted to Dr. David Fulton, M.D., M.R.C.S., L.R.C.P., pathologist and physician to the Peterborough and District Memorial Hospital, and to Dr. L. P. Garrod, M.R.C.S., F.R.C.P., pathologist to St. Bartholomew's Hospital, London, through whose kindness and good offices this interesting specimen was made available to me.

Dr. Fulton sent me the information that the patient was a male, 39

years old, who had complained of severe headaches for a period of 5 years. During one of these headaches he had become comatose and died. He was a native of Peterborough, but at the age of 16 had joined H.M. Royal Navy, and during his service had travelled through the Mediterranean, calling at Gibraltar and Malta and at ports in Spain, France, Italy and Greece. Later he visited the West Indies. He was discharged apparently fit, in 1932, and complained of no indispositions till 1936.

Section of the brain showed severe haemorrhages in the pons Varolii, a lesion which caused his death. The lateral ventricles were somewhat enlarged, but Dr. Garrod tells me that this may have been due to an attack of cerebro-spinal fever at the age of 16. In the posterior horn of the lateral ventricle was situated a well developed and matured coenurus. It seemed to occupy the entire cavity very completely, having grown to fit all the various crevices and diverticula of the situation. In gross structure the coenurus was perfectly typical. It was thin walled and there seemed to be no adventitious cyst. The scolices were very numerous. The exact number cannot be stated here as certain portions of the coenurus had been used for sections, but in the material that remained, there were more than 700. They were arranged in groups of varying sizes—some of the groups contained as many as 70 or 80 scolices, others as few as 10 or 12. Thirty scolices taken at random from various parts of the cyst were examined microscopically for identification and the cyst was shown to be a specimen of *Coenurus cerebralis*.

One rather interesting feature is the large proportion of atypical forms present among the scolices. There were probably more than 50%, for of the 30 examined, 16 were triradiate and one was tetraradiate. They had 6 or 8 suckers : some had 2 rostella and some only one. These atypical scolices did not occupy a well circumscribed area, for often a group of 3 or 4 scolices, closely apposed to each other in space in the cyst would show 2 typical and 2 atypical forms, or 1 typical and 3 atypical ones. They seemed to be scattered at random irregularly among the normal ones. These triradiate forms often carried typical and normal hooks in shape and sometimes in size, but occasionally they were slightly smaller. Here it may be remarked that all the hooks examined from this coenurus tended to be a little smaller than is usually found in this species. The number of hooks on the triradiate forms was usually increased by about 30%. There were furthermore 3 scolices, normal so far as sucker number is concerned, which showed

abnormalities in the rostellum. One was completely unarmed with a very degenerate rostellum while the others were armed but the hooks were ill formed. Some appeared only as blade and guard : others had only irregular masses of chitin to indicate the presence of armature.

Coenurus cerebralis is a well known larva of almost cosmopolitan distribution. In its adult stage, *Multiceps multiceps,* it occurs as a parasite of the small intestine of the dog. The larval stage is typically a parasite of the central nervous system, brain and spinal cord, of various Ungulata. It has been reported from the brain of man only once before—by Brumpt in his Précis de Parasitologie. The conditions of infection seem to correspond very closely with those of the present case. Brumpt however records finding groups of cestode hooks in pockets of the brain tissue and sloughing of the epithelial lining of the ventricular cavity. He assumes therefore that the cyst examined was not the original one. It was either a daughter cyst or else had developed independently from a hexacanth embryo. There was no evidence in the present case that this was not the primary infection.

It is not possible to say where the man had picked up the infection. The parasite has a wide distribution having been recorded from the whole of the Mediterranean basin, where he had travelled. It is common enough too in England. It would not be unreasonable however to suggest from the clinical history that it had been acquired in this country for the headaches, which may have been a symptom of its presence, were first remarked on in 1936—4 years after his discharge from H.M. Royal Navy. *Coenurus* is a fairly quick growing parasite, unlike hydatid and would probably reach a considerable size in the space of a year. This at any rate is the case among coenuri which have been studied by me. *C. serialis,* for example, in rabbits reaches maturity within a few weeks and even when the " strain " is a slow growing one, yet a very large size, with considerable proliferation, is attained in less than a year.

Though this seems to be only the second record of its presence in man, yet it may be of commoner occurrence than has been suspected. For it was only by chance that this parasite was noticed at autopsy and it may well be that other infections have been missed and passed unnoticed.

The specimen has been deposited in the Museum of the London School of Hygiene and Tropical Medicine.

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The Metabolism of Trichinosed Rats During the Early Phase of the Disease.

By W. P. Rogers, M.Sc., Ph.D.

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Trichinosis in man, until recently a problem of little importance in England, has assumed a new status owing to the increased number of cases reported during the last few months. This has led to an increased necessity for better methods of diagnosis and more information on the actual disorders occasioned by the presence of the parasites is also needed, for, although the literature on Trichinosis in general is voluminous, the clinical chemical pathology of the disease appears to have been somewhat neglected. Some work, however, has been carried out on the blood chemistry of infected animals. Thus Hartman et al. (1939 and 1940) have investigated the chemical changes in the blood of men and dogs infected with *Trichinella spiralis* but found little to indicate the nature of host physiological malfunction induced by the parasites. Again, Augustine (1936) reported that infections in men and rabbits did not affect carbohydrate metabolism.

The present paper deals with attempts to define the course of metabolic changes in rats after infection. This work involves the early periods of the disease only and does not embrace all possible lines of investigation. Thus these studies are general in nature and the more specialised examination of liver and kidney function has not been included. Nor, as yet, has work been carried out on the blood changes (except by indirect inference) for it was felt that the withdrawal of blood from the rats would in itself affect the metabolism of the animals thus obscuring changes induced by the parasites. Indeed, the experimental animals were handled as little as possible and efforts were made to ensure that they were not subjected to undue excitement or conditions leading to changes in activity.

General Experimental Procedure.

Four worm-free rats, 2 males and 2 females, each housed in a clean wire metabolism cage which allowed the separate collection of faeces

and urine, were used. The animals were kept on a standard diet (300 parts of finely ground whole wheat, 30 parts of " full cream " dried milk and one part of NaCl) during the period of the experiment. Food was fed in 3 lots of 5 grams each day, except during late in the experiment when this amount was decreased as some of the animals lost appetite. Each lot of 5 grams of food was mixed with 8 ml. of a watery suspension of cod liver oil (100 parts of tap water to 1 part of oil). It was found that this method of presenting the food as a thinnish paste was highly successful for it was almost always quickly eaten and there was no observable wastage.

The faeces from each rat was collected three times a day and stored till a four day sample was available for analysis. Urine was also collected as a four day sample, the catchment trays at the bases of the cages being washed with enough distilled water to bring the urine volumes up to 100 ml. Actually, to allow the rats to become accustomed to the diet, they were fed in the cages for 8 days before commencing the experiment, after which the cages were re-cleaned and the collection of period (4 days) samples of faeces and urine started.

Immediately after collection, toluene was added to the urine which was then stored at 2°C. until required. Each period sample of faeces was thoroughly mixed with distilled water, weighed, a sample for helminth examination removed, reweighed, dried at 100°C. and weighed again. The total dry weights of the period faecal samples could thus be obtained. The dried faeces were then finely ground and samples for analysis prepared.

Every 8 days, two 5 gram samples of food were weighed out. To each the 8 ml. of watery cod liver oil was added and the lot dried at 100°C. After drying, the material was weighed, ground to a fine consistency and samples prepared for analysis.

On the first day of each period the rats were weighed.

Helminthological Procedure.

During the 8 days prior to the commencement of the experiment proper, the faeces of the rats were collected and examined for helminths (by washing and concentrating) and for eggs (by flotation methods). No parasites or eggs were found. Similar examinations were made on samples of faeces from each period during the experiment (see Table 1). At the beginning of the 5th period, rats 2, 3 and 4 had, mixed with their food, 0·40 grams (wet weight) of rat flesh containing approximately 680 *T. spiralis* larvae. Rat 1 was given 0·375 gram of flesh containing

approximately 640 larvae. No wastage was noted.

TABLE 1.

Showing the numbers of *T. spiralis* larvae and males estimated to be passed in faeces during the various periods of the experiment. Figures shown between brackets indicate the actual numbers of parasites counted in the samples of faeces taken for investigation.

Period.	Rat 1♀.	Rat 2♀.	Rat 3♂.	Rat 4♂.
1, 2, 3 and 4 ...	nil	nil	nil	nil
5	53 (8) larvae	94 (13) larvae	22 (4) larvae	5 (1) larvae
6	nil	nil	nil	nil
7	nil	10 (1) larvae 10 (1) males	nil	6 (1) larvae 6 (1) males
8	nil	nil	nil	nil

Six days after the termination of the 8th period, rats 3 and 4 died with intestinal haemorrhage. The pelts were removed from the dead animals and the bodies were minced and digested with artificial gastric juice. Larvae in samples of the digest were counted and the total number of parasites in the rats estimated. Rat 3 harboured 3,200 larvae and rat 4, 4,866 larvae. One week later rats 1 and 2 were killed and the numbers of larvae present estimated as before. From rat 1, 25,600 larvae and rat 2, 28,533 larvae were obtained.

DIGESTION OF CRUDE FIBRE.

Though crude fibre does not form a large proportion of the diet of rats, the digestion of this constituent was examined for Stewart (1932) working with mixed *Trichostrongyle* infestations in sheep and Rogers (unpublished) investigating *Haemonchus contortus* infested sheep, found indications that fibre digestion was impeded by the presence of the parasites. Consequently, crude fibre estimations (by the normal acid and alkali treatment method) on the rat food and faeces were carried out.

The fibre content of the food samples of the various periods did not vary greatly or regularly and hence the average of all the figures obtained was used to determine the amounts of fibre fed at any period. Thus it was considered that, except in instances when the food intake was lowered due to loss of appetite by the parasitized animals, the

fibre fed to the rats did not vary greatly from period to period.

Table 2 summarises the results obtained.

Digestion of Protein.

Protein estimations were made using the Kjeldahl method. Macro digestions were carried out and the digest made up to a standard volume from which a known proportion was taken for micro distillation. This method was found to give similar results to the normal macro method and had the advantage of being shorter and more economical. Micro digestions were not used owing to the fact that there were probably

Table 2.

Showing the digestion of crude fibre in rats before and after infection with *T. spiralis*. The animals were infected at the beginning of the 5th period. For further explanation see text.

Period.	Rat 1♀.			Rat 2♀		
	Intake grams.	Voided grams.	Digested per cent.	Intake grams.	Voided grams.	Digested per cent.
1 ...	1·164	0·948	18·56	1·164	0·995	14·52
2 ...	1·164	0·927	20·36	1·164	0·958	17·70
3 ...	1·164	0·971	16·58	1·164	0·974	16·32
4 ...	1·164	1·057	9·20	1·164	0·969	16·75
5 ...	1·164	0·916	21·31	1·164	1·039	10·74
6 ...	1·067	0·891	16·49	1·164	1·022	12·20
7 ...	0·970	0·833	14·12	1·164	0·930	20·10
8 ...	1·164	0·958	17·70	1·164	0·912	21·65
	Rat 3♂.			Rat 4♂.		
1 ...	1·164	0·991	14·86	1·164	1·026	11·86
2 ...	1·164	0·958	17·70	1·164	—	—
3 ...	1·164	—	—	1·164	1·119	3·87
4 ...	1·164	0·966	17·01	1·164	0·987	15·21
5 ...	1·164	1·107	4·90	1·164	1·043	10·40
6 ...	1·164	1·064	8·59	1·164	0·992	14·78
7 ...	1·164	1·002	13·92	1·164	0·968	16·84
8 ...	1·164	0·958	17·70	1·164	0·989	15·03

minor variations in the constituents of the food and faeces which could be obviated by using large samples for digestion. As in the case of crude fibre, the protein content of the food was found to vary but slightly and irregularly and an average figure was again used for calculating the period rations. It was considered that (total N) × 6·2 gave the protein content of the food or faeces. No allowance was made for the addition of 0·40 grams (wet weight) of flesh to the normal ration in period 5. Actually then, the figure obtained for the percentage digestion for this period was slightly lower than the true result. Table 3 summarises the results obtained.

TABLE 3.

Showing the digestion of protein in rats before and after infection with *T. spiralis* larvae. "Intake" refers to the protein in the food and "Voided," that in the faeces. The animals were infected at the beginning of the 5th period. For further explanation see text.

Period.	Rat 1♀.			Rat 2♀.		
	Intake grams.	Voided grams.	Digested per cent.	Intake grams.	Voided grams.	Digested per cent.
1 ...	6·417	1·34	79·11	6·417	1·34	79·12
2 ...	6·417	1·18	81·61	6·417	1·05	83·64
3 ...	6·417	1·17	81·76	6·417	1·24	80·68
4 ...	6·417	1·38	78·50	6·417	1·14	82·24
5 ...	6·417	1·29	79·89	6·417	1·30	79·75
6 ...	5·882	1·28	78·42	6·417	1·42	77·88
7 ...	5·347	1·26	76·44	6·417	1·47	77·10
8 ...	6·417	1·31	79·59	6·417	1·36	78·81
	Rat 3♂.			Rat 4♂.		
1 ...	6·417	1·12	82·55	6·417	1·19	81·46
2 ...	6·417	1·17	81·77	6·417	1·01	84·27
3 ...	6·417	1·40	78·19	6·417	1·28	80·06
4 ...	6·417	1·26	80·37	6·417	1·07	83·33
5 ...	6·417	1·38	78·50	6·417	1·09	83·02
6 ...	6·417	1·34	79·12	6·417	1·31	79·59
7 ...	6·417	1·46	77·25	6·417	1·48	76·94
8 ...	6·417	1·33	79·28	6·417	1·19	81·46

TABLE 4.

Showing the daily Nitrogen balance of rats before and after infection with *T. spiralis*. The figures given are averages for the four days of each period. "Output" refers to the urinary N output. Animals were infected at the beginning of the 5th period. For further explanation see text.

Period.	Rat 1♀.			Rat 2♀.		
	Intake mgs.	Output mgs.	Balance mgs.	Intake mgs.	Output mgs.	Balance mgs.
1 ...	203	158	+45	203	156	+47
2 ...	209	174	+35	215	157	+58
3 ...	210	157	+53	207	179	+28
4 ...	201	148	+53	211	165	+46
5 ...	203	159	+44	204	158	+46
6 ...	184	129	+55	200	144	+56
7 ...	163	141	+22	198	143	+55
8 ...	204	149	+55	202	161	+41
	Rat 3♂.			Rat 4♂.		
1 ...	212	136	+76	209	141	+68
2 ...	210	126	+84	216	156	+63
3 ...	201	119	+81	205	142	+63
4 ...	206	118	+88	214	145	+69
5 ...	201	137	+64	213	166	+47
6 ...	203	103	+100	204	137	+67
7 ...	194	145	+49	197	143	+54
8 ...	203	144	+59	209	165	+44

DAILY NITROGEN BALANCE.

Nitrogen in the urine was found by the Kjeldahl method, 10 ml. of urine being digested, the digest brought to a volume of 100 ml. by the addition of distilled water and 5 ml. samples of this being distilled. Table 4 shows the calculated daily nitrogen balances. The figures

giving the balance for period 5 are slightly low because no allowance was made for the addition of flesh to the ration. The " Intake " shown in the table gives the nett N intake per day calculated by subtracting the total N per day in the faeces from the total N per day taken in with the food.

Excretion of Creatinine and Creatine.

Creatinine in the urine was estimated by the Folin (1914) method. Since the urine was very dilute it was necessary to use 10 ml. for each determination. Some difficulty was experienced in estimating " total creatinine." Benedict's (1914) boiling down method was found to give very low results, probably owing to the destruction of creatinine due to the high temperatures used. Further, the modification introduced by Eggs and Vanoli (1935) to overcome this was found to give somewhat variable results. In consequence, the Folin (1914) autoclave method (using 5 ml. of urine) was used. This method gave consistent results similar to those listed by Brody et al. (1934) when the female rats were examined but the figures for the male rats were naturally somewhat lower. Brody et al. (1934) do not state the sex of the animals to which they refer in their tables. It must be noted, however, that exactly the same technique, when applied to normal human male urine gave creatine figures which were somewhat high. Before " total creatinine " estimations were carried out it was ascertained that the urine was free from glucose.

The results are shown as mgs. of preformed creatinine per day per kg. rat weight and mgs. of " total creatinine " per day per kg. rat weight in Graphs 1,2, 3, 4, 5, 6, 7 and 8.

Protein in the Urine.

Urinary protein was estimated by Folin's gravimetric method, using 10 ml. samples of urine. It was considered that haemoglobin was not present in the urine as no haemochromogen bands could be detected spectroscopically (even in long columns of urine) after the addition of alkali and sodium hydrosulphite. It is probable, therefore, that the protein found in the rats' urines was largely albumen. Table 5 shows the average daily albumen excretion for each period of the experiment.

TABLE 5.

Showing the average daily amount of protein (mgs.) found in the rats' urine for each period of the experiment. Rats were infected at the beginning of the 5th period.

Period.	Rat 1♀.	Rat 2♀.	Rat 3♂.	Rat 4♂.
1	17	25	10	25
2	10	12	15	50
3	12	—	20	60
4	15	10	25	57
5	20	35	37	101
6	22	12	35	100
7	32	10	42	62
8	—	30	55	70
9	25	52	37	75

URINARY AMMONIA AND UREA.

Urinary ammonia and urea were estimated by the Van Slyke and Cullen (1916) aeration and titration technique. Though special precautions were taken to refrigerate the urine with toluol as soon as possible, it was usually some time before determinations could be carried out and the ammonia estimations cannot be accepted without reserve. It was for this reason that ammonia : titratable acid ratios were not determined.

The results as the average daily output of ammonia and urea N in mgs. for the various periods are shown in Graphs 11, 12, 13, 14, 15, 16, 17 and 18.

RAT WEIGHTS.

The experimental animals were weighed on the first day of each period. Table 6 shows the results obtained. It can be seen that growth was delayed in the period when infective material was fed. The flesh containing the *T. spiralis* larvae was fresh and small in quantity (0·40 grams. wet weight was the maximum amount used) and it is therefore considered that the loss in weight was due to the infective agents only.

It may be noted here that the fall in weights at the termination of

the experiment in some part caused the steep rise in the creatine and creatinine output recorded for periods 9 and 10 (figures are given per kg. rat weight, see Graphs 1, 2, 3, 4, 5, 6, 7 and 8). However, there was a large absolute increase in the creatine and its anhydride excreted during those periods.

TABLE 6.

Rat weights in grams. The animals were infected at the beginning of the 5th period.

Period.	Weights in grams.			
	Rat 1.	Rat 2.	Rat 3	Rat 4.
1	100	107	106	109
2	112	118	124	125
3	114	124	129	125
4	122	127	143	137
5	118	124	143	135
6	129	136	160	150
7	125	144	163	155
8	133	143	167	160
9	135	150	163	154
10	130	145	150	140

DISCUSSION OF RESULTS.

The proportion of the larvae recovered from the faeces of the rats immediately after infection may be taken as an indication of the resistance of the animals. It would thus appear that the males were less resistant than the females (see Table 1). (It should be noted that the male and female rats were of two different strains and there is no reason why the different reactions of the animals should be attributed to sex alone.) The fact that the males died with intestinal haemorrhage, not unusual among *T. spiralis* infected rats (Cameron, 1934, page 138) whereas the females lived, also indicated a lower resistance in the former animals. It therefore appears reasonable to class rats 1 and 2 as " resistant " animals and rats 3 and 4 as " non-resistant " animals.

No consistent indications of the effects of *T. spiralis* on the digestion of crude fibre in either resistant or non-resistant rats were obtained. Apparently fibre digestion fell in rats 2 and 3 after infection and in all the animals there was an increase during the last period (see Table 2). The latter result is similar to that obtained when protein digestion was being examined (see Table 3). These indefinite conclusions are paralleled by those of Stewart (1932) who found that fibre digestion was affected by Trichostrongyle parasites in some of his experimental lambs but not in others.

During the first 4 days after infection, protein digestion was apparently undisturbed (see Table 3). In the 6th period the efficiency of digestion fell slightly and in the 7th period it reached the lowest point in all rats. During the latter period rats 2 and 3 developed a moderate diarrhoea and rat 1 lost appetite. The figures in Table 3 indicate that the non-resistant rats suffered most from disturbed digestion.

Possible factors causing decreased efficiency in digestion of protein may have been as follows :—

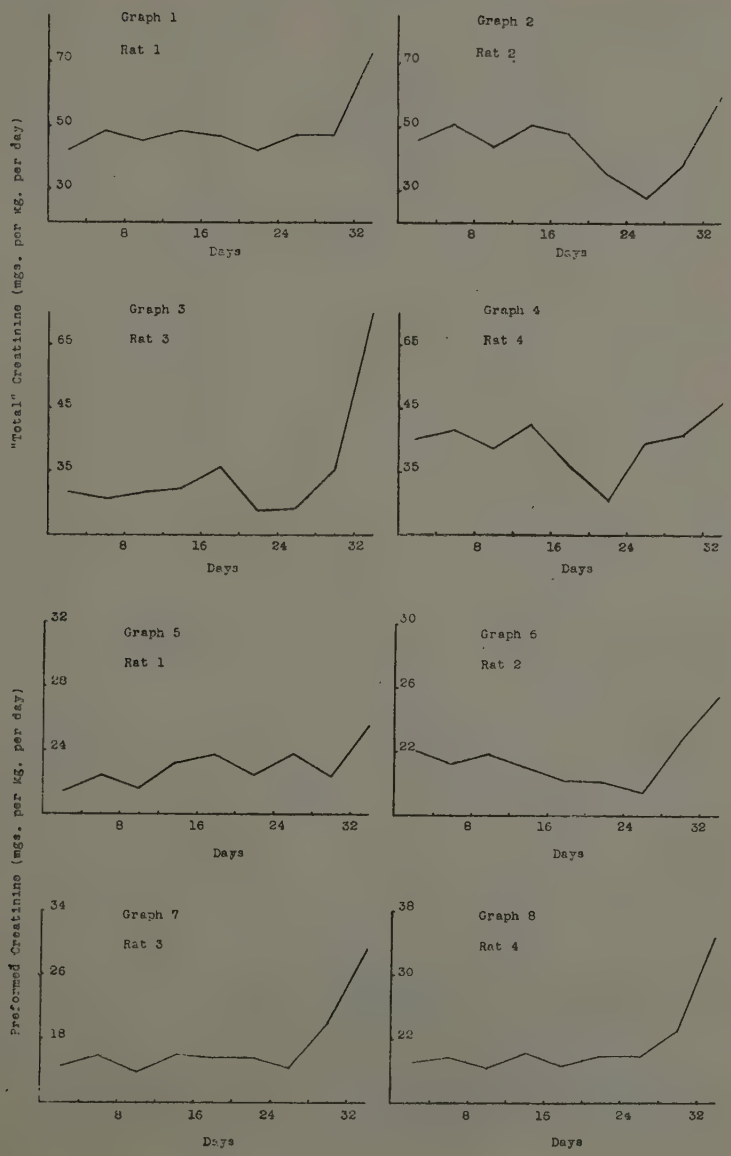
I. Antiproteases secreted by the adult parasites.

Hamil (1906) and Harned and Nash (1932) found that *Ascaris lumbricoides* secreted antitryptic enzymes. Other workers (Stewart, 1932a and Rogers, unpublished) have found similar antienzymes in Trichostrongyle parasites of sheep. It may be thought, therefore, that *T. spiralis* secretes antienzymes and these may have been responsible for the results observed. However, Faust (1939, page 361) suggests that the female Trichinella invade the host intestinal mucosa on the 5th day after infection. Thus it would appear that antiprotease would not be secreted in the lumen of the host intestine after this time and hence it is unlikely that digestive disturbance during the 6th and 7th periods could be due to antienzyme. If, however, the majority of the adult females did not enter the host tissue till early in the 7th period, the

LEGENDS.

Graphs 1, 2, 3 and 4 showing the daily (given as averages taken over 4 day periods, in mgs. per kg. rat weight) excretion of " total creatinine " by rats infected with *Trichinella spiralis* 16 days after the commencement of the experiments. Rat 1 (♀) was fed slightly fewer larvae than rats 2 (♀), 3 (♂) or 4 (♂).

Graphs 5, 6, 7 and 8 showing the daily (given as averages taken over 4 day periods, in mgs. per kg. rat weight) excretion of preformed creatinine by rats infected with *Trichinella spiralis* 16 days after the commencement of the experiments. Rat 1 (♀) was fed slightly fewer larvae than rats 2 (♀), 3 (♂) or 4 (♂).


Graph 1
Rat 1
70
50
30
8
16
24
32
Days
Graph 2
Rat 2
70
50
30
8
16
24
32
Days
"Total" Creatinine (mgs. per kg. per day)
Graph 3
Rat 3
65
45
35
8
16
24
32
Days
Graph 4
Rat 4
65
45
35
8
16
24
32
Days
Graph 5
Rat 1
32
28
24
8
16
24
32
Days
Graph 6
Rat 2
30
26
22
8
16
24
32
Days
Preformed Creatinine (mgs. per kg. per day)
Graph 7
Rat 3
34
26
18
8
16
24
32
Days
Graph 8
Rat 4
38
30
22
8
16
24
32
Days

antiprotease may have been in part responsible for the lowered digestion of protein in the 6th period.

II. Damage to the host intestinal mucosa by the female parasites.

There can be no doubt that the irritation caused by the movement of the worms in the host tissues would disturb digestion but, in order to give the observed results, it must be considered that most adult migration took place early in the 7th period, i.e. about 9 days after infection.

III. Damage to the host intestinal mucosa by the release of larvae.

This factor may be ignored as it is evident that the maximal numbers of larvae were deposited in the intestinal lymphatics much later than the 7th period. Evidence to support this contention is as follows :—

a. Rats examined about the 10th period contained 3,000 to 5,000 larvae. Several days later it was found that rats 1 and 2 harboured 25,000 to 29,000 larvae. Hence largest numbers of larvae were deposited after the 9th period.

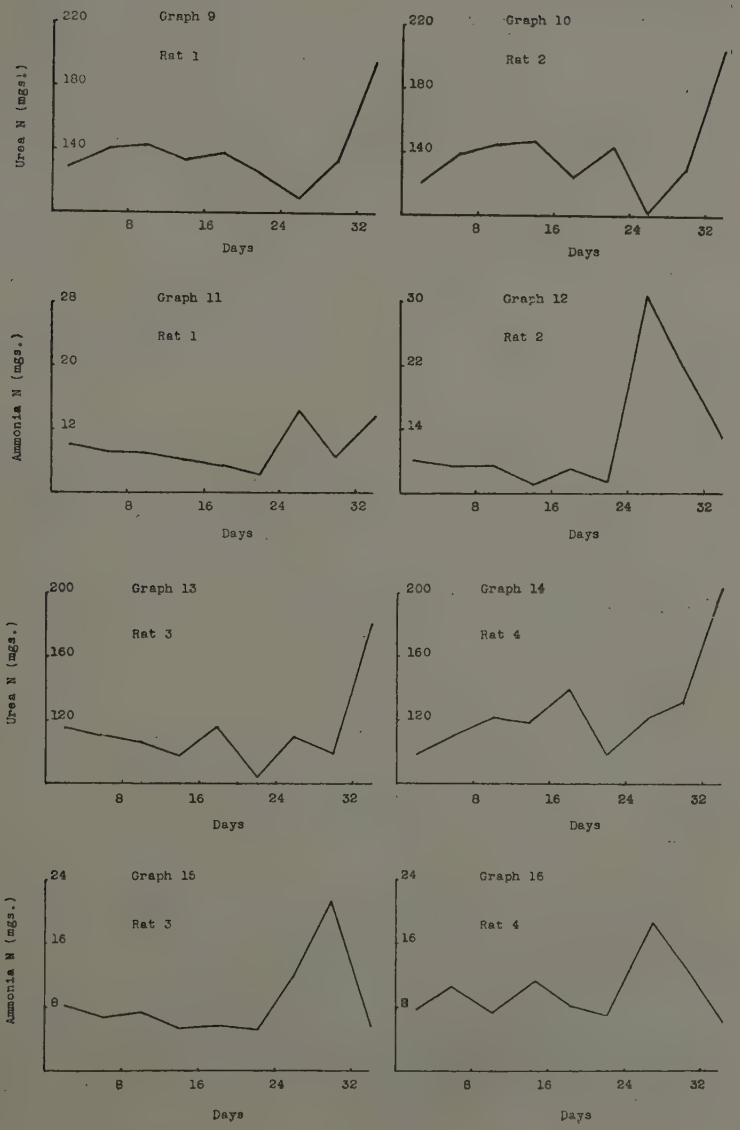
b. Rats 3 and 4 died during the 10th period. Apparently death was due to intestinal haemorrhage caused by the release of large numbers of larvae in the tissues.

In reviewing these possibilities it seems most probable (in spite of the contradiction inferred from Faust's (1939) review) that digestive disturbances resulted from a combination of the 1st and 2nd factors, the initial lowering in the percentage of protein digested being due to antienzymes and the greatest fall being caused by both antienzyme and mechanical damage arising from the migration of the adult worms. The sudden return to normal conditions in the 8th period would then be brought about by the abrupt termination of the secretion of antiprotease in the intestinal lumen and the rapid repair of the damaged mucosa.

The irregular lowering of the N balance after infection was probably partly due to the fall in N intake. In rates 3 and 4, however, there were indications that there was an absolute increase in urinary N during

Graphs 9, 10, 11 and 12 showing the daily (given as averages taken over 4 day periods, in mgs. of N) excretion of urea and ammonia by rats 1 and 2 infected with *Trichinella spiralis* 16 days after the commencement of the experiments. Rat 1 (♀) was fed slightly fewer larvae than rat 2 (♀).

Graphs 13, 14, 15 and 16 showing the daily (given as averages taken over 4 day periods, in mgs. of N) excretion of urea and ammonia by rats 2 and 3 infected with *Trichinella spiralis* 16 days after the commencement of the experiments. Both rats were males and received approximately the same number of infective larvae.


Graph 9
Rat 1
Urea N (mgs.)
220
180
140
8
16
24
32
Days
Graph 10
Rat 2
220
180
140
8
16
24
32
Days
Graph 11
Rat 1
Ammonia N (mgs.)
28
20
12
8
16
24
32
Days
Graph 12
Rat 2
30
22
14
8
16
24
32
Days
Graph 13
Rat 3
Urea N (mgs.)
200
160
120
8
16
24
32
Days
Graph 14
Rat 4
200
160
120
8
16
24
32
Days
Graph 15
Rat 3
Ammonia N (mgs.)
24
16
8
8
16
24
32
Days
Graph 16
Rat 4
24
16
8
8
16
24
32
Days

some periods after infection. The absolute rise in the urinary N of these rats during period 5 may be taken as indicating toxic destruction of protein (Peters and Van Slyke, 1931, page 296) for it was probably caused by an increase in urea + ammonia excretion (see Graphs 13 and 14). Since tissue invasion by larvae did not occur till long after the 5th period, it appears that the young adult parasites themselves must have an immediate toxic effect on the host. The absolute increase in the urinary N of rats 3 and 4 during period 8 was again probably due to urea but this may have been attributable, not to adult parasites, but to tissue destruction by masses of larvae. It is notable that toxic effects were most marked in " non-resistant " animals.

The most constant feature in the excretion of urinary urea was the fall in output during the 7th period i.e. 8 to 12 days after infection, see Graphs 9, 10, 12 and 13). Factors causing this fall may have been as follows :—

(a) *The fall in protein digestion.*

Since the decrease in urinary urea was, to some extent accompanied by a fall in creatinine, it may be thought that the lowered urea output was due to the decreased absorption of protein in the intestine (Jolliffe and Smith, 1931, 1931a) which took place simultaneously. However the fall in N intake was usually less than 10 mgs. per day whereas the urinary urea N increased by 3 or 4 times that amount. The lowered ingestion of protein could, therefore, only be partly responsible for the reduced urea output.

(b) *Liver Dysfunction.*

It seems reasonable to disregard this factor for the power of urea formation by the liver is only markedly reduced when liver destruction is very great (Mann, 1927) and in all the infected rats examined in this experiment urea output was only reduced for a very short period.

(c) *Acidosis.*

It has been maintained that, during acidosis, urea is destroyed in the kidney, giving rise to ammonia (Benedict and Nash, 1921 and 1926). Since the fall in urea was, in these experiments, accompanied by an increase in ammonia (it should be noted that the fall in urea N was not compensated by the fall in dietary N intake + the increase in ammonia N) acidosis may be considered to have been the casual factor. However, Schneller (1935) and Krebs (1936) deny that urea is the precursor of urinary ammonia and furthermore the ammonia curves obtained (see

Graphs 11, 12, 15 and 16) were not similar to those normally found in acidosis.

(d) *Damaged renal vessels or faulty cardiac function.*

A reduction of the pressure of the blood in the kidneys would reduce the glomerular filtration rate and this would lead to decreased urea excretion. However, any reduction in the glomerular filtration rate is usually reflected by a fall in the creatinine output, a fact in variance with the present results.

(e) *Oligurea causing increased urea reabsorption in the kidney tubules.*

Though it cannot be stated that oligurea occurred during the 7th period of the experiment (the urine was always collected in a standard volume of water) oligurea does occur in severe cases of trichinosis in man. It is possible, therefore, that the urine volume fell below the " augmentation limit " during the acute period giving rise to the results obtained.

Preceding the fall in urea excretion during period 7, a marked rise in output occurred immediately after infection in the " non-resistant " rats (see Graphs 13 and 14). This was probably due to the toxic destruction of body proteins and indicates that the young adult parasites elaborate toxins having severe effects on the non-resistant host. It follows that heavy infestations with *T. spiralis* may cause marked disorders before larvae are present in the host tissue.

In all experimental animals there was an increase in urinary urea after the 7th period (see Graphs 9, 10, 13 and 14). This rise was far greater than that necessary to compensate for the previous fall and, occurring at a time when larvae commenced to migrate through the host tissue, was probably due to toxins from (a) damaged host tissue, (b) larvae and (c) adult parasites in the intestinal mucosa. Factor (a) was probably of most importance in this connection.

The excretion of ammonia followed a fairly normal course till towards the end of the experiment when a sudden rise was noted (see Graphs 11, 12, 15 and 16). That this was in response to acidosis seems unlikely for during recovery from acidosis increased ammonia excretion persists for a considerable period. Although the rise in urinary ammonia occurred in coincidence or a little after the fall in urea excretion, it must not be inferred that the excess ammonia was formed by the hydrolysis of urea but rather that it was due to an early deflection of N in intermediate metabolism (Smith, 1937, page 180).

Preformed creatinine was excreted in fairly normal quantities until

late in the experiment when a very slight retention followed by a rise in the excretion rate was noted (see Graphs 1, 2, 3 and 4). Creatine excretion showed the same changes to a marked extent (see Graphs 5, 6, 7 and 8). Interpretation of these results is difficult for information on the metabolism of creatine and its anhydride is scarce. As figures relating to the blood are, at present, lacking it is not clear whether the changes in creatine output were due to variations in the hosts' general metabolism or to kidney disorder. In the latter case, it would appear that the disturbance, during the 7th period, was located in the tubules rather than the glomeruli, for creatine, which showed little change, is secreted largely by glomerular filtration in mammals whereas creatine may be reabsorbed or excreted by the tubules (Smith, 1937, page 109). The increase in the excretion rate following the 7th period may have been, in the case of creatine, wholly due to the previous retention, but muscular damage caused by the migrating larvae may have contributed, especially in the excretion of creatinine.

It is worthy of note that Markowicz and Bock (1931) examining patients infected with *T. spiralis* found that during the acute febrile stage a reduction of urinary creatine and creatinine occurred. They also stated that in all fatal cases there was a rapid premortal increase in creatine.

Summary.

1. The changes in protein digestion, crude fibre digestion and the chief urinary constituents found in four rats after experimental infection with *Trichinella spiralis* are detailed.

2. Diarrhoea and anorexia occurred during the period 8 to 12 days after infection when protein digestion fell to its lowest point. It is suggested that this was due to antiproteases secreted by the adult parasites and to mechanical damage in the intestinal mucosa caused by their movements.

3. Urinary N rose immediately after infection in "non-resistant" rats. This was followed by a period of decreased N output after which the excretion rate rose steeply.

4. The urea output also rose immediately after infection in "non-resistant" rats. During the period 4 to 12 days from the time of infection urea excretion fell. Thereafter there was a great increase in its rate of output. Most of these changes have been attributed to toxins elaborated by the adult parasites for massive larval invasion of

the tissues probably did not occur till after the experiments were terminated.

5. Ammonia excretion rose as urinary urea decreased. The fall in urea N was not compensated by the rise in ammonia N + the fall in dietary N intake. The ammonia output returned rapidly to normal suggesting that the excess ammonia was not produced in response to acidosis when the urea excretion rate rose.

6. Creatine excretion showed a marked fall during the period 4 to 12 days after infection. Urinary creatine and creatinine rose steeply following this period. Possible reasons for these variations are discussed.

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On the morphology of *Mermithonema entomophilum* n.g., n.sp., a nematode parasite of the fly, *Sepsis cynipsea* L.

By T. Goodey, D.Sc.

In the body cavity of a female specimen of the fly, *Sepsis cynipsea* L., (Diptera-Cyclorrhapha), caught at this Institute in August, 1930, the writer found eight adult nematode parasites; 3 females and 5 males. The fly was dissected in Ringer's solution in which the worms became actively motile when set free from the host. At the time of dissection it was seen that the fly's ovaries were in an undeveloped state and there is no doubt that this was due to the presence of the parasites within the body cavity. Beyond noting the fact that the worms seemed to be of a mermithid type they were not investigated further at the time but were fixed in hot 70% glycerine alcohol, to which a trace of Nile Blue sulphate solution was added, and were set aside. Although a good number of the flies were dissected during the month of August no other example was found to be infected with this species of nematode and, as the worms have not been found again since 1930 in casual specimens of the fly captured and dissected here, it seems desirable to describe them since they appear to be new to science. Structurally they bear some resemblance both to *Aproctonema entomophagum* Keilin, 1917 and to *Tetradonema plicans* Cobb, 1919 but differ from these species in several anatomical features. Their systematic relationships are discussed in detail later on.

Anatomy.

Dimensions :—*female*, lengths, 7·45 mm., 9·21 mm., and 9·26 mm., $a = 72$, 76 and 77, V = 48·8%, 52·2% and 48·6% respectively; *male*, length, 2·88 mm. to 3·81 mm., $a = 33·5 - 40$, $\beta = 3·94 - 4·9$, $\gamma = 24 - 24·9$, mean dimensions of the 5 specimens, length, 3·28 mm., $a = 36·8$, $\beta = 4·45$, $\gamma = 24·5$, spicule, 125μ long.

As the three females had the ovaries so fully developed as to occupy most of the body the internal anatomy has been studied in detail in the male worms where the gonads did not obscure the structures belonging to the alimentary tract.

In both sexes the body is of almost uniform width throughout most

of its length but tapers a little towards the head and the tail in the female whilst in the male the tail is strongly curved ventrally and ends in a point. The cuticle is rather thin and is coarsely annulate. The individual annules appear to be of about the same width but differ from the striae occurring on many other nematodes in that they do not maintain a regular arrangement in their course over the body. In one of the females they were almost entirely absent. As they pass over on to the lateral surfaces of the body they take on an irregular appearance and are not interrupted by any lateral field. Winged lateral lines appear to be absent.

Beneath the cuticle the body wall carries longitudinal markings which appear to be either longitudinal striae or, more probably, are the attachments to the hypodermis of the numerous cells composing the musculature of the body wall. On the dorsal and ventral sides of the body these cells form extensive fields projecting into the body cavity. Criss-cross fibres such as occur in the cuticle of certain species of Mermis have not been seen.

The head is somewhat roundly conical in shape and in some specimens is flat or slightly depressed on the anterior face. It does not seem to be offset from the body by any distinct constriction although, in one or two specimens, the first annule of the body appeared to be rather prominent and to reveal the backward extent of the head.

Circumoral papillae have not been seen but there appear to be 6 rather inconspicuous head papillae. In one male worm these papillae showed fairly clear indications of a central core. The mouth is central and leads into a simple tube which traverses the oesophagus. It is slightly expanded in what may perhaps be termed the buccal cavity but immediately assumes a uniform diameter of about 2·5μ which is maintained throughout its length. The outer wall of the oesophagus is very indistinct in most of the specimens and it is difficult to delimit it clearly from the surrounding tissues of the body. It is, in fact, much less clearly defined than is suggested by the lines in the drawings. The nerve ring occurs at approximately 0·11 mm. from the anterior end of the body and the oesophagus narrows a little in passing through it. An excretory pore could not be found.

A short distance behind the nerve ring there occurs the first and most clearly distinguishable of certain granular structures which are associated with the œsophagus throughout the rest of its course. The first granular body has the appearance of a large cell. It lies above and below the œsophagus but in what manner is attached to it, it has

been impossible to determine clearly. Its contents have the appearance of rather coarse granules of uniform size and the whole organ stained green with Nile Blue sulphate. A fairly large, rather dense nucleus was observed in it.

Behind this cell the oesophagus extends on a more or less wavy course for a further 0·45 mm. to 0·47 mm., gradually becoming more and more indefinite in outline; the walls surrounding the central lumen also taking on a rather broken and indistinct appearance. Associated with this region there appear to be a further 7 degenerate cellular bodies. They are recognisable chiefly by their rather elongated nuclei; separating cell walls between them being only faintly discernible here and there. A certain amount of granular protoplasm occurs round each nucleus. In figs. 1 and 5 these degenerate cells are represented as lying rather to one side of the oesophagus but in all 5 of the male worms the cells appeared to lie above and below the oesophagus; their exact connection or relation to it could not, however, be clearly determined even under high magnification. The oesophagus appears to end vaguely just in front of the forward end of the anterior testis. In none of the worms was there found any indication of a median or terminal oesophageal bulb or swelling neither was it possible to distinguish transverse muscles or muscle fibres within the walls of the oesophagus.

What is the nature of the granular cells or to what structure in other nematodes can they be considered to be analogous? In the case of such indistinct and apparently degenerate structures it is possible only to conjecture an answer. It seems reasonable, however, to suggest that the whole organ corresponds to what Steiner (1933) calls the stichosome of other mermithids which, in this case, is composed of 8 stichocytes. In the case of *Agamermis decaudata*, parasitic in grasshoppers and other insects in U.S.A., Christie (1936) has described and illustrated the structure of the stichosome, consisting of 16 nucleate stichocytes, in the young parasitic stages of the worm. Chitwood (1935) also has demonstrated in the case of *Agamermis decaudata* that each stichocyte is an oesophageal gland with a duct opening from it into the lumen of the oesophagus. In the present case, doubtless owing to the degenerate condition of the cells, as the worms are fully adult, it has not been possible to distinguish any ducts. If the stichosome is essentially a larval organ subserving some function in connection with the nutrition of the worm in its early parasitic stages of growth, this may account for the degenerate appearance of the organ in *Mermithonema entomophilum*.

It has been mentioned above that the oesophagus ends vaguely. It seems to rest upon but not to be continuous with an organ which corresponds to the intestine, namely the trophosome. In all the male specimens this could be traced alongside the oesophagus and stichosome ending blindly in the vicinity of the first stichocyte. It extends backwards throughout the length of the body and apparently ends blindly close to the terminal part of the vas deferens. No connection can be traced between it and the opening of the ejaculatory duct and there is no rectum or anus. Structurally it has a somewhat vacuolate appearance and there are scattered nuclei in its walls. There is no discernible lumen in it and it is probably syncytial in character.

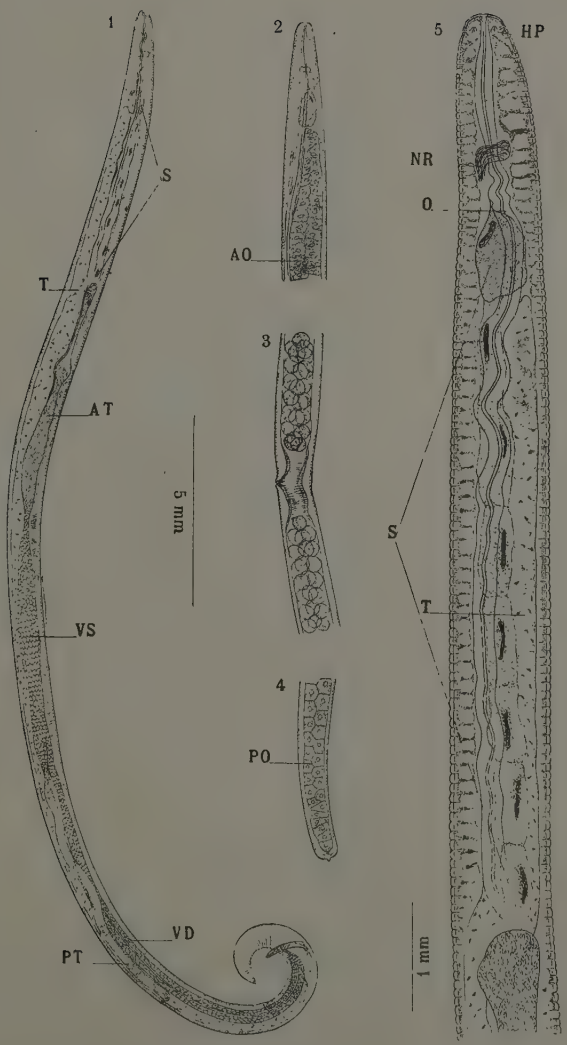
Male.—All the males found were fully mature sexually and had well developed gonads. The testes are paired and outstretched anteriorly and posteriorly and each is crowded with extremely small sperms having the appearance of short rods. The forward testis narrows down a little before joining the stout-walled vescicula seminalis which at its widest region occupies quite threequarters of the body width and finally gives off on its dorsal side the posterior testis. The latter extends backwards, overlying or running alongside the vas deferens, and generally ends in a bluntly rounded end some distance in front of the papillate tail region. The vas deferens lies on the ventral side of the body. It has stout cellular walls and finally tapers to a narrow ejaculatory duct which opens to the surface at the orifice through which the spicule is extruded. The body wall of the tail region is richly supplied with lateral muscles which extend from some distance in front of the spicule to the tip of the tail. In addition, there is a group of muscles arising from the dorsal body wall and inserted at, or a little in front of, the spicular orifice. There is a single, large, hollow, horn-like spicule which in lateral aspect has the shape shown in figure 6. The open anterior end is offset by constriction and the shaft tapers gradually to a rather blunt point. It is surrounded by a sheath of muscles which are attached to the head end and are

Fig. 1.—Male worm in lateral aspect to show general shape and structure.

Figs. 2, 3 and 4.—Anterior end, middle region and tail end, respectively, of a female worm drawn to same scale as fig. 1.

Fig. 5.—Oesophageal region of a male worm, in lateral aspect, showing relation of stichosome, composed of 8 stichocytes, 7 of which are degenerate, to the oesophagus.

AO, anterior ovary, AT, anterior testis, HP, head papillae, NR, nerve ring, O, oesophagus, PO, posterior ovary, PT, posterior testis, S, stichosome, T, trophosome, VD, vas deferens, VS, vescicula seminalis.



Mermithonema entomophilum n. g., n. sp.

inserted around the spicular orifice, mainly posterior to it. Special retractor muscles, such as are found in many male nematodes attached to the head of the spicules and inserted anteriorly in the dorsal body wall, have not been found. It is probable, therefore, that the muscle sheath surrounding the spicule somehow functions both as a protractor and a retractor system.

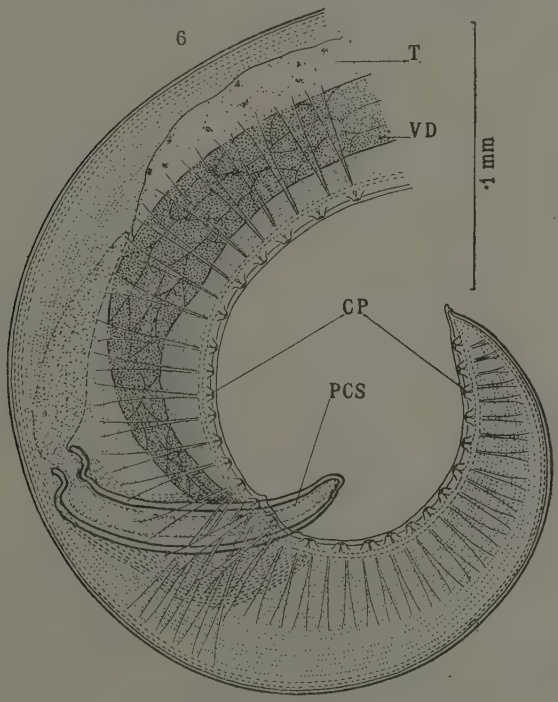
Within the cavity of the spicule is a core of rather dense protoplasmic material which is continued out of the head of the organ into a more or less fusiform mass. It appears to be attached to the inside of the tip of the spicule but not to the lateral walls of the shaft. After issuing from the head of the organ the substance lies free in the body cavity without discoverable attachments to the body wall. In two specimens it was drawn out and tapering as shown in fig. 6, but in two others it had the form of a rather short rounded mass. On its ventral side the substance has a somewhat fibrillar appearance suggestive of muscle tissue and for a time the writer has inclined to regard the whole mass as a retractor muscle with an inner attachment to the spicule. As, however, no attachment to the dorsal body wall could be found, the idea of it having a muscular function was abandoned. As far as the writer is aware, nematode spicules do not possess a protoplasmic core and it is very difficult to suggest what the nature and function of the mass can be in the present case. Possibly it represents the remains of the tissues from which the spicule was formed but if this is so the substance is remarkably free from any signs of being in a degenerated condition.

The ventral surface of the body in the tail region is furnished with a series of small papillae which extend from some distance in front of the spicule aperture to the tip of the tail. Each side of the body has a row of 25 to 28 of these small papillae sub-ventrally placed. Each papilla has the shape of a rather squat, truncate cone, is crateriform in structure and has a very fine central core.

Female.—The female tail is bluntly rounded with, in one case, the suggestion of a very small terminal process in the cuticle. In all three females the two ovaries were well developed and outstretched in the body, the anterior one extending close up to the first granular stichocyte and the posterior one reaching to within a short distance of the tip of the tail. The vulva is equatorial in position. Its lips are rounded and the very short vagina opens into a common cavity having muscular walls which is made up from the ends of the two uteri. Each of the latter blends imperceptibly with its ovary; no intervening constricted oviduct being discernible. Each uterus is crowded with eggs which are round

in outline and are about 50μ in diameter. As no free eggs were seen in the body cavity of the fly it is impossible to state in what condition of segmentation they are laid.

Biology.—It is impossible to offer any observations on the course of the life history of the parasite as only one fly was found infected. The



Mermithonema entomophilum n. g., n. sp.

Fig. 6.—Male tail in lateral aspect. CP, caudal papillae, PCS, protoplasmic core of spicule, T, trophosome, VD, vas deferens.

method of escape of the female worms from the host's body is unknown but it seems probable, from analogy with the life history of *Aproctonema entomophagum* and *Tetradonema plicans*, that the gravid females make their way out of the host and in so doing probably bring about its death. How the host becomes infected can only be conjectured. Either the embryonated eggs are swallowed by the fly larvae, as

appears to be the case with *Tetradonema plicans*, or the embryos, after hatching, make their way into fly larvae by direct penetration from the outside, as is the case in *Aproctonema entomophagum*, the embryos of which possess a mouth spear. Whichever mode of entry into the host is followed the parasites finally come to lie in the body cavity of the fly where they persist throughout its metamorphosis and in which they bring about sexual sterilisation by arresting the development of the gonads.

Systematics.

Only two mermithid nematodes, the adults of which occur within the host's body cavity, have previously described namely, *Aproctonema entomophagum* Keilin, 1917, and *Tetradonema plicans* Cobb, 1919. Both of these were found in the larvae, pupae and imagines of flies belonging to the genus Sciara; the former in *Sciara pullula* Winn, and the latter in *Sciara coprophila* Lintner. A further paper on *Aproctonema entomophagum* by Keilin & Robinson (1933) deals with its morphology and life history whilst Hungerford (1919) published some biological observations on *Tetradonema plicans*. It will be sufficient to discuss the relationship of the new parasite to these two nematodes in order to arrive at its systematic position and the chief differential features of the three species are set out below.

Mermithonema entomophilum n.sp.—Length, female, 7·45 mm. to 9·26 mm., male, 2·88 mm. to 3·81 mm. Mouth simple, leading to plain tube traversing oesophagus. Associated with latter an 8-celled stichosome. Intestine represented by a syncytial trophosome. Anus absent. Ovaries paired, outstretched; oviduct absent. Eggs round, 50μ in diameter. Female tail blunt, round. Male tail pointed, strongly flexed ventrally. Spicule single, large, 125μ long. On each side of tail a sub-ventral row of 25-28 small crateriform papillae.

Tetradonema plicans.—Length, female 5 mm., male 0·8 mm. Mouth simple, lumen of oesophagus tubular. Associated stichosome a "tetrad" of four cellular bodies. Intestine a blind trophosome. Anus absent. Ovaries paired, reflexed. Eggs shaped like mushroom caps, 33μ in diameter. Female tail tapering, pointed. Male tail pointed and flexed ventrally. Spicule single, 32μ long. Caudal papillae absent.

Aproctonema entomophagum.—Length, female, 4·4 mm. to 6 mm., male, 1·9 mm. Buccal spear present. Lumen of oesophagus tubular.

No special stichosome present. Intestine of many large cells round central core-like strand. Anus absent. Ovaries paired and outstretched. An oviduct present between each ovary and uterus. Eggs spherical, 90μ in diameter. Tail blunt, round. Male tail rounded but bearing a small terminal knob. Spicule single, 90μ long. Caudal papillae absent.

It is clear from a consideration of the foregoing data that the newly described worms differ from *Tetradonema plicans* and *Aproctonema entomophagum* in size, in the oesophagus with its associated stichosome, in the size of the eggs, in the size of the spicule and especially in the presence of numerous small papillae on the male tail. It becomes necessary, therefore, to erect a new genus, *Mermithonema*, for their reception.

Mermithonema n. g.

Females, 7·45 mm. to 9·26 mm., males 2·88 mm. to 3·81 mm. long. Body typically worm-like in shape. Cuticle with transverse annules, lateral lines absent from cuticle. Mouth leading into plain tube traversing simple, non-muscular oesophagus; associated with latter a series of 8 cell-like bodies, probably representing the stichosome of true Mermis species. No direct connection between end of oesophagus and the syncytial trophosome which represents the intestine. Anus absent, excretory pore absent. Female with paired, outstretched ovaries; vulva equatorial, eggs rounded in outline, 50μ in diameter. Male with paired, outstretched testes, spicule single, 125μ long. Tail strongly flexed ventrally, tip pointed. On each side a sub-ventral row of 25 to 28 small crateriform papillae.

Genotype *Mermithonema entomophilum* n. sp.

Parasitic in body cavity of a female example of the fly, *Sepsis cynipsea* L., caught at the Institute of Agricultural Parasitology, Winches Farm, St. Albans, England. Life history unknown.

The new genus should probably be considered, along with the closely related genera, Aproctonema and Tetradonema, as belonging to the family Mermithidae. If a separate family is needed for these three genera they should be placed in the Aproctonematidae of Keilin and Robinson.

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Observations on a giant race of the stem eelworm, *Anguillulina dipsaci*, attacking broad beans, *Vicia Faba* L.

By T. Goodey, D.Sc.

(*From the Institute of Agricultural Parasitology, St. Albans.*)

Debray and Maupas (1896) described a disease of broad beans, *Vicia Faba* L., occurring in and around Algiers, N. Africa, caused by the stem eelworm, *Anguillulina dipsaci*, the adults of which were considerably larger than from any other host plant attacked by this species; the females being 1·758 mm. to 2·216 mm., and the males 1·716 mm. to 2·016 mm. long. As far as the writer is aware, from the time their paper appeared to the present time no further record of such giant forms of *A. dipsaci* has been published. During the past few years the writer has encountered similar large forms in diseased broad bean stems, first in material originating in Portugal and on two later occasions in bean plants grown under ordinary field conditions in this country, and it seems desirable to put on record a short account of his observations.

Material.

In the Spring of 1933 Prof. F. T. Brooks, F.R.S., of the Botany School, Cambridge, sent to the writer several pieces of broad bean stem showing a diseased condition. A further supply of similarly affected

pieces was also received from the same source early in 1934. The material had originally come from Portugal where, according to Prof. Brooks's letter, a disease of broad beans exhibiting the symptoms shown by these pieces is of common occurrence. The stems were discoloured, some areas being pale yellowish green and others reddish to dark brown.

Irregular swellings or flattish galls occurred in the region of the nodes and extended on to one or more of the four sides of the stem. The epidermis in the affected areas appeared inflated and on lifting pieces of it along with the underlying soft parenchyma and examining in water under a microscope numbers of eelworms were readily found. It was at once apparent that the adult worms were exceptionally long and on killing by heat and making drawings under the camera lucida several were found to have a length of about 2 mm. Males and females as well as eggs and larvae occurred in the galled tissues. A good number of adult specimens were killed in hot 5% formalin and after washing in water were transferred to dilute glycerine and finally mounted in glycerine. Measurements and dimensions were made by means of camera lucida drawings and gave the following results.

Female, length, 1·73 mm. to 2·23 mm., $\alpha = 50-64$, $\beta = 7-12$, $\gamma = 15{\cdot}8-20$, $V = 76\%-84\%$, average of 22 specimens, length, 1·97 mm., $\alpha = 58{\cdot}2$ $\beta = 9$, $\gamma = 17{\cdot}5$, $V = 81{\cdot}9\%$. *Male,* length, 1·51 mm. to 1·93 mm., $\alpha = 58-74$, $\beta = 6-8{\cdot}4$, $\gamma = 14{\cdot}6-19{\cdot}1$, average of 23 specimens, length, 1·77 mm. $\alpha = 67$, $\beta = 7-8$, $\gamma = 16{\cdot}9$.

These figures agree fairly well with those of Debray and Maupas for large forms except as regards the alpha proportion which they give as 35-41 for females and 52-61 for males; that is to say the worms examined by the writer are on the whole slenderer than those measured by the French authors. Apart from this, the other proportions and the relative position of the vulva in the females are in agreement and this, taken in conjunction with the fact that the signs of disease as described by them agree with those found by the writer, there is little doubt that the same giant race of the parasite was concerned with the production of disease in beans both in Algiers and in Portugal.

A point worthy of note is that in the affected bean stems examined by the writer only large examples of the parasite occurred whereas Debray and Maupas found adults of two sizes, i.e. smaller forms measuring 1 mm. to 1·5 mm. long as well as the larger forms attaining a length of about 2 mm. The smaller worms occurred in small numbers in the vicinity of reddish or purple spots on the sides of the

stems generally close to the base and without any accompanying swelling of the cortical parenchyma. The large forms were plentiful in the blister-like swellings of the nodes and internodes as well as in other situations such as swollen leaf stalks. They suggest that the difference in size observed between the adult worms found under these two disease conditions may have been due to the environment in the former case being relatively less favourable to growth which was manifested in the smaller size attained by the worms. Whether this is a probable explanation of the remarkable difference in size shown by these two forms appears doubtful to the writer. It seems more likely that two distinct races of different average size were encountered in the diseased Algerian beans since, as will be shown later in this paper, even on unfavourable hosts the large forms still maintain their size.

Debray and Maupas also reported that on diseased bean plants from the vicinity of Amiens, France, adult worms found in an affected pod i.e. under conditions very favourable to growth, were of normal size; the longest males being 1·444 mm. and the longest females 1·615 mm. long. These figures may be compared with some measurements made by the writer of adults from diseased bean stems from Yorkshire. This material was kindly supplied by Mr. L. R. Johnson, Department of Agriculture, The University, Leeds. Seven males ranged in length from 0·94 mm. to 1·16 mm. and five females from 1·05 mm. to 1·57 mm. Although but few worms were measured it was clear that they were not giant forms but were of the normal size attained by the adults parasitizing oats, clover, potatoes, narcissus, etc.

It has already been mentioned that the giant race has been found on two occasions on diseased beans growing under ordinary field conditions in this country. In May, 1937, the writer received some pieces of diseased bean stem from a crop grown on a farm in Hertfordshire. The stems were twisted, swollen at the nodes and the epidermis was inflated. The stipules were swollen at their junction with the stem and several of the leaves were infected, being much swollen in the short leaf stalk. The plants were in the flowering stage and the parasites were found in the swollen base of the calyx. Adult worms were abundant in all the affected regions and were of large size as the following measurements show. Four males ranged in length from 1·64 mm. to 1·77 mm. the average length being 1·68 mm. whilst 11 females varied in length from 1·46 mm. to 2·15 mm. the average length being 1·81 mm.

Early in June, 1938, a sample of diseased tick beans from a field in

Kent were sent to the writer by Mr. S. G. Jary, South-Eastern Agricultural College, Wye. The crop was also affected by " chocolate spot " a disease of fungal origin. Very much the same kind of disease symptoms were found on these plants as on those already described. In the gall-like swellings of the stem numbers of large *A. dipsaci* were found. Five males varied in length from 1·64 mm. to 1·89 mm., the average being 1·70 mm. and 8 females ranged in length from 1·53 mm. to 1·78 mm., the average length being 1·72 mm.

It is evident from these two records that the giant race of the parasite may occasionally occur on beans in this country. In neither of the cases, however, is the origin of the infection known i.e. whether it came from a previous crop, from weeds or from a seed-borne infection of the beans sown. Since the giant race has been recorded only from broad beans, it is feasible to suggest that the parasite may have been introduced with the seed; a suggestion which would have some weight if it could be shown that broad bean seed is imported into this country from N. Africa or Portugal; the only two countries at present known as homes of this race of the parasite. It is not clear, however, from the enquiries which the writer has made that field and tick beans are brought in from abroad for seed purposes. On the contrary, they are apparently raised for seed mainly in England.

The question is incapable of further solution at the present time. More information is clearly required on the incidence of stem eelworm disease on broad beans in this country coupled with the determination of the size of the parasite before one can say definitely whether the giant race is indigenous on this crop in England.

Infection Experiments.

Using the pieces of diseased bean stem received from Prof. F. T. Brooks as an inoculum, attempts were made to set up infection in broad beans and in various other plants. The object of these experiments was to study the disease on broad beans and, as far as possible, maintain it on this host. In addition it was hoped to test the range of leguminous hosts susceptible to infection and at the same time to determine whether on host plants other than the broad bean the adult worms would attain the same size as on their original host.

Broad Beans.—In April, 1933, on a small plot measuring 12 feet long by 4 feet wide three drills were drawn. In two of them pieces of diseased bean stems were distributed whilst the third row received no inoculum. Broad bean seeds were sown about 6 inches apart in all

three rows and the drills were covered in. During May some of the young bean plants in the two inoculated rows showed evidence of infection as lightish yellow swellings and twistings of the short leaf petioles close to their attachment to the stem. In some cases this was accompanied by blackened areas at the base of the leaf-blade the latter often hanging down limp and flaccid. In some seedlings the uppermost crown of leaves was small and incurved. Some of the stems showed blister-like swellings close to the nodes on one or more of the stem faces and generally close to soil level. Many of the leaves showed creamy yellow leaf blotches a few millimetres in diameter scattered irregularly over the surface. On teasing out the tissues surrounding such spots in water and examining under the microscope the parasites were found in fair numbers. Numerous adult worms were later collected from affected part of the seedlings, were killed by heat and then examined under the microscope and measured for length. 14 males ranged in length from 1·38 mm. to 2·05 mm., the average length being 1·71 mm., whilst 13 females ranged in length from 1·70 mm. to 2·23 mm. and had a mean length of 1·98 mm. It was clear from these figures that on broad beans the large size of the parasite is maintained.

The infected plants were finally lifted and dried off with a view to the stems being used as a further source of infection on the plot in the following year. In 1934 the dried stems were cut up into small pieces and distributed in three drills situated in the same positions as the rows drawn in 1933. In row 1 broad bean seed was sown, in rows 2 and 3 seeds of the following were sown in 3 feet lengths : onions, garden peas, field peas, vetches, oats, rye, barley and wheat. None of the plants growing in rows 2 and 3 showed any sign of infection in the form of lesions or swellings of stem and leaf structures and only a few of the bean plants in row 1 showed a few swellings at the base of the stem below soil level when the plants were well grown. One or two typical large-sized adult worms were found in these lesions but it was apparent that, for some reason unknown, the infection resulting from the use of the stems harvested in 1933 was extremely light. Further attempts to carry on infection on broad beans in 1935 on the same plot failed and the strain was lost.

Other Legumes.—In 1933 and 1934 pot experiments were set up in which soil was inoculated with small pieces of diseased bean stem amongst which the seeds were sown. A control uninoculated pot was sown in each case. Seeds of the following were sown :—*Lathyrus odoratus* L. (sweet pea), *Pisum sativum* L. (garden pea), *P. arvense* L.

(field pea), *Phaseolus vulgaris* L. var. *humilus* (kidney bean, Canadian wonder), *Phaseolus coccineus* L. (scarlet runner), *Vicia sativa* L. (vetch), *Anthyllis Vulneraria* L. (kidney vetch), *Trifolium pratense* L. (red clover), *T. hybridum* L. (alsike clover), *T. repens* L. (wild white clover), *T. incarnatum* L. (crimson clover), *Medicago denticulata* Willd. (burr clover), *M. lupulina* L. (nonsuch), *Glycine Max* Merr. (soy bean), *Lupinus luteus* L. (lupin), *Onobrychis sativa* Lam. (sainfoin).

Only in the case of field pea, garden pea, soy bean and sainfoin were a few lesions produced with a slight invasion of the underlying tissues; all the other plants tested remained free from infection.

Pisum arvense L. (field pea).—In between the two terminal leaf stalks of a seedling about 2 inches high a small brown lesion of the epidermis was found. On carefully dissecting this region in water under the microscope several specimens of *A. dipsaci* were found in the parenchyma. Of the 19 adults obtained, 11 were females and 8 males. The females varied in length from 1·745 mm. to 2·12 mm., the mean length being 1·925 mm. The males varied in length from 1·53 mm. to 1·91 mm., the mean length being 1·77 mm.

Another seedling had two leaflets which were rather irregular in shape and had small surface lesions on them. On dissecting the tissues in and around these lesions one or two dead and shrivelled examples of the parasite were found. On further close examination of the stem of this plant a brown lesion was found on a short leaf stalk and a little further down the stem in between a stipule and the stem itself, another brown surface lesion was found. Careful dissection of these revealed several adult examples of the parasite amongst the parenchyma cells. The worms were picked up, killed by heat and measured by means of line drawings made under the camera lucida. 16 females ranged in length from 1·47 mm. to 1·97 mm., the mean length being 1·71 mm. 11 males varied in length from 1·35 mm. to 1·9 mm. the mean length being 1·61 mm.

Pisum sativum L. (garden pea).—On one seedling a small irregular swelling was found close to the tip of a leaflet. This was dissected in water and 10 specimens of the parasite were obtained, 3 were adults and the rest larvae. Of the former, two females measured 1·70 mm. and 1·86 mm. whilst the single male measured 1·82 mm. Another rather weakly seedling was found on close examination to have a few brownish epidermal lesions on a short leaf stalk and a lesion on the

upper side of the midrib of the leaf blade. By dissection of these areas a number of adult worms were obtained. 13 females varied in length from 1·42 mm. to 2·18 mm., the mean length being 1·76 mm. 12 males varied in length from 1·44 mm to 1·98 mm., the mean length being 1·73 mm.

It was observed that though the parasites had reached the adult stage none of the females appeared to have been inseminated or to have eggs in the uterus. There was no invasion of the leaf and stem tissues apart from the areas in the immediate vicinity of the epidermal lesions and it would seem that this host was not really congenial to the parasites.

Glycine Max Merr. (soy bean).—On one small seedling at the edge of the leaf blade close to the short leaf stalk a small brown lesion was found. The tissues below this were rather thickened and tough and on dissection there were found 13 adults of the parasite and 12 pre-adult larvae. Of the adults, 11 females ranged in length from 1·275 mm. to 1·965 mm., the mean length being 1·64 mm. The 2 males measured 1·25 mm. and 1·375 mm., the mean being 1·312 mm. Another small leaf with an irregular thickening of the edge of the leaf blade but lacking any brown discoloration was found on dissection to contain 7 dead and shrivelled examples of the parasite. Among the young leaves at the crown of this plant there were also found 4 dead and shrivelled specimens on the outside of the tissues. Soy bean was apparently an uncongenial host for the parasite which, though able to effect a certain small amount of local injury to the young tissues was unable to establish itself and produce major symptoms of disease.

Onobrychis sativa Lam. (sainfoin).—A few seedlings showed small brown epidermal lesions in the region of the stipule of the first true leaf at its junction with the stem. In the tissues under one of these 9 adult worms were found. 5 females ranged in length from 1·53 mm. to 1·76 mm., the mean length being 1·67 mm. and 4 males varied in length from 1·44 mm. to 1·64 mm., the mean length being 1·49 mm. In four other seedlings showing small brown lesions numbers of larvae varying from 6 to 22 were found. In two cases they appeared to be rather shrivelled and poorly developed. The adults, though belonging to the large race, did not attain a length of nearly 2 mm. as did some of those found in the lesions on the three previous hosts.

It would seem that even on rather unsuitable hosts such as these four species of legumes proved to be and on which small lesions only were caused by the parasite, the latter did not become smaller but kept its

typical size. From this it may be inferred that the size of the adult worms is not a response to some nutritional factor but is a genetic characteristic of the strain or race.

Comparison of the Giant Race from Beans with a Normal Sized Race from Oats.

It was considered desirable to make a comparison between the lengths of adults of the large race from beans and a race of more normal size and to treat the measurements statistically. Unfortunately for this purpose large numbers of a normal sized race from broad beans were not available so use was made of measurements of adult worms from "tulip root" oats of which an abundant supply was at hand. A typically diseased oat plant was teased up in water and the invading nematodes were floated out from the swollen leaf bases. Fully adult males and females were picked up at random, mounted in drops of water, killed by heat and then measured for length by making line drawings under the camera lucida. 50 males and 50 females were measured in this way. Of the large forms from broad beans measurements of length were obtained from 37 males and 35 females.

Table I.

Lengths of adults of oat and bean races of *A. dipsaci*.

Race.	Sex.	Mean ± St. err.	St. dev.	Coeff. var.	n.
Oat	Female	1·4912±0·01075 mm.	0·076395 mm.	5·123%	50
	Male	1·3828±0·01227 mm.	0·086781 mm.	6·276%	50
Bean	Female	1·9711±0·02635 mm.	0·15588 mm.	7·908%	35
	Male	1·7335±0·02490 mm.	0·15149 mm.	8·739%	37

The relevant data are set out in the accompanying table. The difference between the mean lengths expressed as a ratio to its standard error is given by the formula :—

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sigma} \sqrt{\frac{n_1 n_2}{n_1 + n_2}},$$

the value for which is for females 20·04, and for males 14·96, showing that both differences have a very high significance. In fact, for all practical purposes it is impossible that the two samples could have

been drawn from the same population of lengths. Only the extremes from each sample, amounting to about 2% of the total, show any overlap. Statistical treatment thus confirms the impression obtained from observation of the large size of the giant race of the adults from broad beans and one is therefore justified in concluding that it is a true and distinctive race.

A point which it seemed worthwhile to determine was whether a normal sized race, such as that causing " tulip root " in oats, would manifest any tendency to an increase in length if transferred to broad beans as a host. It is known that in Yorkshire both oats and broad beans suffer from attacks of a strain of *Anguillulina dipsaci* capable of causing disease in both crops. Pots inoculated with dried " tulip root " oat material and sown with broad beans gave rise to plants which were infected and showed swollen lesions of the stem and leaf stalks. From these it was possible to obtain considerable numbers of adult male and female worms. These were picked up at random, killed by heat and afterwards measured for length. 85 males and 82 females were measured in this way. The data are given in Table 2.

TABLE II.

Lengths of adults of an oat race of *A. dipsaci* infecting beans.

Sex.	Mean ± St. err.	St. dev.	Coeff. var.	n.
Female	1·384±0·01555 mm.	0·14084 mm.	10·176%	82
Male	1·328±0·01373 mm.	0·095634 mm.	7·201%	85

So far from the results revealing any tendency of the parasite to an increase in length on the broad bean they show that there is actually a slight decrease in length in both sexes.

The writer is greatly indebted to his colleague, Dr. B. G. Peters, for his help in carrying out the statistical treatment.

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On the occurrence of the chrysanthemum eelworm, *Aphelenchoides ritzema-bosi*, in a tomato fruit.

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The occurrence of the chrysanthemum eelworm, *Aphelenchoides ritzema-bosi* (Schwartz, 1911), in an unusual situation, namely within the tissues of tomato fruits, calls for brief report since, though well known as a leaf parasite of a good number of plants, it has not, to our knowledge, been reported previously from a fruit.

Towards the end of October, one of us (S.H.B.) visited a grower who complained of having a number of tomato fruits from which the calyx tissue came away at the slightest touch. These fruits then rotted deeply towards the centre. Two such fruits were taken from the store for examination in the laboratory. One of them was attacked by the fungus *Didymella Lycopersici* whilst the other was found to contain eelworms. This fruit was sent by S.H.B. to T.G. with a request for a determination of the species of eelworm present and an opinion on whether it was a parasitic species or not.

On receipt of the fruit the senior author cut it in two by a cut passing along the longitudinal axis from the calyx region and through the central fleshy placenta. It was then seen that the discoloured diseased area was about $\frac{1}{4}$ in. deep by about $\frac{5}{8}$ in. broad and occupied much of the solid fleshy base of the fruit. On teasing up a small portion of the diseased tissues in water and examining under the binocular microscope, considerable numbers of small eelworms were found together with numerous eggs and larvae in various stages of growth. Several of the adult worms were collected, mounted in a drop of water and, after killing by gentle heat, were further examined when it was found that they were specimens of *Aphelenchoides ritzema-bosi* (Schwartz, 1911), the chrysanthemum eelworm. The tails of the male worms were strongly flexed ventrally into the characteristic curve and the spicules

were of the right shape and size for this species. No other species of nematode was found in the affected flesh nor was there any sign of fungal hyphae. Each half of the fruit was placed in a glass container having a little water in it in order to keep the atmosphere moist. After two days small portions of the tissue were taken from various regions and were examined for the presence of eelworms. It was then found that the nematodes had penetrated throughout most of the flesh since adults, eggs and larvae were found in portions of tissue taken from just beneath the skin directly opposite the calyx region where there were no signs of disease or discoloration. They also occurred in the soft material of the mesocarp around the seeds. A secondary infection of some kind of saprophytic yellowish soft-rot bacteria was already present in the diseased area when the fruit was received and this gradually spread into the tissues of each half causing a thorough decay in them.

Report that the eelworms had been identified as *Aph. ritzema-bosi* was sent to S.H.B. who visited the grower again and collected about 50 discarded tomato fruits which were then examined for the presence of eelworms. In only four of these fruits were eelworms present. In three they occurred in small numbers but in the fourth there were large numbers of adults and eggs. In this fruit also bacteria were present and it seems quite possible that they were introduced in both cases by the eelworms.

The plants which had borne these fruits had been pulled up so that it was impossible to make any observations as the presence of leaf-blotch or other symptoms or to determine whether eelworms were present in the leaves. The grower explained that the plants had been grown out of doors. There had been no chrysanthemums grown on the ground before them neither were there any chrysanthemums growing in proximity to them. The plants were apparently healthy during growth though a few of them had pale or discoloured lower leaves. It is thus impossible to form any precise ideas as to the probable source of the eelworm infection.

Aph. ritzema-bosi as a parasite of the tomato plant has been reported only once previously by Junges (1938) who transmitted it experimentally to the tomato from heavily infected gloxinia leaves and found that the tomato leaves showed characteristic symptoms of disease within 14 days. He also maintained the infection on tomato for a second year. In addition, he lists the tomato as a host showing a natural

infection with *Aph. ritzema-bosi* in a garden at Leipzig-Kleinzschocher. There can be no doubt, therefore, that the tomato plant can be parasitized more or less severely by this species of eelworm but there has, hitherto, been no record of it invading and causing disease in the tomato fruit. The present findings show that the fruit can become infected and manifest symptoms of disease. In the present instance a plant or two must, presumably, have been infected with the eelworms some of which finally reached the calyx region and then entered the fruits.

The tomato plant is already known as a host of certain parasitic nematodes which affect both root and shoot. The root knot eelworm, *Heterodera marioni* (Cornu) Goodey, causes serious disease to the tomato by the production of root galls on plants grown under greenhouse conditions in this country and out of doors in warmer climates. The potato root eelworm, *Heterodera rostochiensis* Woll. (generally referred to as the potato race of *H. schachtii*) is at the present time becoming of increasing importance as a parasite of tomato roots in commercial practice especially at certain centres in this country. The meadow nematode, *Anguillulina pratensis* (de Man), can invade tomato roots and produce small necrotic areas in them though it is not so serious a parasite as the two species of Heterodera. Williams (1936) found extensive spongy swellings of the stem and leaf-stalks of young tomato plants to be heavily infected with the stem eelworm, *Anguillulina dipsaci*, and was able to reproduce these disease symptoms experimentally by spraying the stems of other young tomato plants with a watery suspension of the eelworms obtained from the crushed infected material. In a later note Williams (1937) reported the successful infection of tomato plants by spraying with a suspension of eelworms from diseased narcissus bulbs. From Junges's findings, already cited, it is clear that tomato leaves are susceptible to attack by yet another eelworm, namely, *Aph. ritzema-bosi* and the present note shows that this species is capable of entering and of causing disease in the flesh of the tomato fruit. Though there is no evidence in the present case that the tomato plants had obtained their eelworm infection from chrysanthemums yet it is a not unreasonable conjecture that the infection was probably derived from diseased chrysanthemum leaves or from some other infected ornamental. Possibly the plants were very mildly parasitized when quite young as they were purchased from a nurseryman who is a flower grower and there is thus the possibility of their having been infected at source. On the practical side, therefore, it may be recommended that all dried infected chrysanthemum leaves

(which are capable of harbouring the eelworm in a viable condition for many months) should be carefully collected and burnt and should on no account be mixed into a compost heap or with soil in which tomatoes are to be grown.

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